ORIGINAL RESEARCH ARTICLE

Current Research on Biosciences and Biotechnology **4** (2) 2023 283-288

Current Research on Biosciences and Biotechnology

www.crbb-journal.com

Development of analysis method of andrographolide from *Andrographis paniculata* using UPLC-PDA

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ABSTRACT

Andrographis paniculata is an herbaceous plant belonging to the family Acanthaceae dan also known as the "King of Bitters". It has been utilized as traditional medicines by the local people in India and Southeast Asia. Many studies have been reported regarding the pharmacological activities of *A. paniculata* and Andrographolide. Currently, in addition to compound isolation and bioactivity assays, validation of analysis method is also required. Validation is used to demonstrate that the method used is appropriate and the results are precise and accurate. The method used needs to be simple, concise, precise, accurate, and widely used. The analysis in this study was carried out using Ultra-Performance Liquid Chromatography because the instrument operates at high pressure. In addition, the system uses fine particles which can reduce column length as well as solvent and time consumption. This study aims to validate analytical methods in determining the contents of andrographolide in an *A. paniculata* Leaves. The analytical method was validated through a Waters Alliance UPLC System with photodiode array (PDA) detector. The *A. paniculata* extract and standard solutions of Andrographolide were analyzed using reverse-phase C18 column which was maintained at 40°C. A mobile phase of 0.1% formic acid in acetnotirile and 0.1% formic acid in water was used at flow rate of 0.3 mL/min to achieve gradient elution. The linearity of the andrographolide showed excellent results (R^2= 0.9999) in the concentration range of 7.8–250.0 µg/mL. The LOD and LOQ values of andrographolide were 0.068 µg/mL and 0.205 µg/mL, respectively. The intra-day and inter-day relative standard deviation (RSD) and relative error (RE) accuracy values of andrographolide were <±15. The quantitative analysis found that *A. paniculata* extract contained 12.45 ±0.06 mg/g of Andrographolide. This developed UPLC-PDA method was proven to be precise, specific, sensitive, and accurate for routine quality assessment of raw material of *Andrographis paniculata* leaves content.

Article history:

Received 08 Feb 2023
Revised 26 Feb 2023
Accepted 27 Feb 2023
Available online 28 Feb 2023

Keywords:

Andrographis paniculata
andrographolide
method validation
UPLC-PDA

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DOI: 10.5614/crbb.2023.4.2/I9YWKM9X

1. Introduction

Andrographis paniculata is an herbaceous plant belonging to the family Acanthaceae (Jayakumar et al., 2013), which is also known as the "King of Bitters". It is a native plant in India and Sri Lanka, also distributed in Southeast Asia, China, America, and the West Indies. This plant also grows well in almost all types of soil (Chauhan et al., 2019). For centuries, *A. paniculata* has been utilized as traditional medicine by the local people in India and Southeast Asia, including Indonesia, to treat all kinds of fever. In Thailand and Malaysia, it is used for the treatment of diabetes and hypertension (Intharuksa et al., 2022). In addition, the plant has been used for the treatment of dyspepsia, influenza, dysentery, malaria, respiratory infections, and stomach pain (Chauhan et al., 2019). Many studies have been reported regarding the pharmacological activities of *A. paniculata*, including anti-inflammatory (Zou et al., 2016; Sheeja et al., 2006), analgesic (Thakur et al., 2015), anti-Sars-Cov-2 (Sa-ngiamsuntorn et al., 2021), antioxidant (Low et al., 2015; Sheeja et al., 2006),

antihypertensive (Zhang and Tan, 1996; Trilestari et al., 2015), antidiabetic and antihyperlipidemic activities (Nugroho et al., 2012).

The increasing knowledge about the effects of plant secondary metabolites on health causes the need for the importance of developing new techniques to separate the components of these bioactive compounds, not only extraction methods but also methods of analysis of bioactive components need to be developed. Several studies of qualitative and quantitative determination of the main bioactive compounds in Andrographis paniculate have been widely reported, such as the use of high-performance liquid chromatography with DAD detection (HPLC-UV/DAD) has been used in these studies. Nevertheless, there are significant disadvantages associated with using the analysis component of HPLC, namely that it requires a relatively lengthy period of time to run a chromatogram and consumes substantial quantities of solvents. UPLC represents a novel approach in the field of liquid chromatography that operates on the same principle as HPLC but with some important advancements. UPLC offers improved runtime and sensitivity due to its utilization of particle sizes of less than 2 µm. The separation process in UPLC is carried out under very high pressure, up to 100 MPa. This results in a reduction in the cost of reagents with a shorter run time as compared to conventional HPLC. UPLC can be regarded as a new direction for liquid chromatography. It improves in three ares speed, resolution and sensitivity, in this system fine particles are used i.e., less than 2.5µm so decrease the length of column, it saves time and reduces solvent consumption (Suryavanshi and Rajasekaran, 2021).

The knowledge of traditional medicine is passed down from generation to generation (Hossain et al., 2014). However, this practice had begun to get eroded and distorted due to continuous invasions and cultural adaptations. One reason is the absence of validation of the process used. Current interest is no longer based solely on the isolation of active compounds and investigation of their bioactivities, but also on the validation of analysis methods that play an important role in controlling or guaranteeing the quality of a product (Tiwari et al., 2010).

Validation is one of the keys to fulfill the requirement of the current good manufacturing practices (CGMP) dan good laboratory practices (GLP) (Chikabanjar et al., 2020). The validation of the analytical method used must align with its intended purpose, and the outcomes derived from it should be employed to evaluate the quality, dependability, and uniformity of the analysis results (Eldin, 2011; Kazusaki et al., 2012).

In validating the analytical method, the method used must be simple, inexpensive, the time required is short, precise, accurate and can be used widely (Tiwari et al., 2010). One such method is Ultra-Performance Liquid Chromatography (UPLC). UPLC is a new and modern chromatographic technique in analytical separations of which working principle is similar to that of HPLC, but with significant improvement in speed, sensitivity, and resolution (Sheliya and Shah, 2013; Sangale and Bhangale, 2021). UPLC instrumentation operates at significantly higher pressures than HPLC instrumentation. Additionally, UPLC employs fine particles with a size of less than 2.5 µm, and the mobile phase flows at a greater speed, resulting in a reduction in column length, solvent usage, and time consumption compared to HPLC (Naresh et al., 2014). This is why the UPLC method is used in the validation of the analysis method.

The major active compound contained in the leaves of *A. paniculata* is Andrographolide (Jayakumar et al., 2013; Churiyah et al., 2015). Andrographolide is a labdane diterpenoid that is used in 26 Ayurvedic formulations for the treatment of liver disease (Pandey and Rao, 2018). Andrographolide has been reported to exhibit various pharmacological activities, such as antioxidant (Low et al., 2015), analgesic (Thakur et al., 2015) anti-inflammatory (Thakur et al., 2015; Low et al., 2015), anti-Sars-Cov-2 (Sa-ngiamsuntorn et al., 2021), immunostimulant (Puri et al., 1993), anticancer (Vetvicka and Vannucci, 2021), as well as antidiabetic and antihyperlipidemic (Nugroho et al., 2012). This study aims to report the isolation of Andrographolide from *A. paniculata* extract. In addition, the validation of analysis methods in determining the contents of Andrographolide in an *A. paniculata* extract was also carried out.

2. Materials and methods

2.1. Chemicals and reagents

Deionized water was purified using a Milli-Q laboratory water purification system (Millipore, Bedford, MA, USA). All reagents and solvents were analytical and HPLC grade (Merck).

2.2. Plant materials

Andrographis paniculata plant was obtained from West Java, Indonesia. The leaves were cut into small pieces and dried in oven at 50°C for 48 h.

2.3. Sample preparation

The dried samples were ground into powder and passed through a sieve (20 mesh). The dried A. paniculate leaves (1 g) was then extracted with MeOH (25 mL) using Ultrasound Assisted-Extraction (UAE) for 20 min at 400C, followed by centrifugation at 4000 rpm for 5 min. The supernatant was filtered using 0.2 µm PTFE filter to obtain the *A. paniculata* leaves extract.

2.4. Thin layer chromatography (TLC) profiling

The TLC plates were prepared by using silica gel GF254 with a length 8 cm and a width of 2 cm. The mobile phase is prepared by making a solvent ratio hexane:ethyl acetat (8:2). The plates were visualized in a UV at wavelength 254 nm, 366 nm and sprayed with sulfonic acid 10%.

2.5. Instrumentation

Analysis was performed on an Accquity UPLC H-Class system equipped with binary solvent manager, sample manager, and column heater (Waters, Milford, Michigan, USA) coupled triple quadrupole. The analyst software Masslynx operated the instrument and executed the data analyses.

2.6. Chromatography system

The mobile phase consisted of solvent A: 0,1% formic acid in water and solvent B: 0,1% formic acid in acetonitrile. The elution was

performed in gradient mode during a time course of 15 min as follows: 0-1 min, 10-20% B; 1.0-2.0 min, 20-35% B; 2.0-5.0 min, 35-60% B; 5.0-8.0 min, 60-80% B; 8.0-8.50 min, 80-100% B; 8.50-15 min, 100% B at flow rate 0.3 mL/min. Finally, the composition was returned to the initial (10% B) in 0.01 min. The reverse-phase C18 column used in the analysis had dimensions of 2.1 × 100 mm ID and 1.7 µm particle size and was configured to detect wavelengths at 230 nm for optimized analysis. The flow rate was set at 0.3 mL/min, the injection volume was 3.0 µL, and the column temperature was maintained at 40°C during the operation.

2.7. Method validation

Validation of the proposed method was performed which includes Specificity, linearity, precision, accuracy, limit of detection (LOD), and limit of quantitation (LOQ) (EMA, 2011; Hwang et al., 2017; Lee et al., 2021; LoBrutto and Patel, 2007; Syukri et al., 2015)

2.8. System suitability

Six replicate injections of the sample sennoside B at a concentration of 125 μg/mL in the methanolic solution was simultaneously analysed. Retention time (Tr). peak area (AUC). tailing factor (T). and theorical plat number (N) were determined. Relative standard deviation (RSD) values of these parameters were calculated to evaluate the system suitability of the developed method. The suitability test was accepted when the RSD values of these parameters were less than 2% (USP. 2009).

2.9. Specificity

In analytical chemistry, specificity pertains to the ability of a method to accurately quantify individual components in a complex mixture. In this study, specificity was evaluated by comparing the chromatograms and PDA spectral patterns of standard solutions, a blank solution consisting of MeOH, and the *A. paniculata* extract (Hwang et al., 2017; Lee et al., 2021).

2.10. Linearity and range

Standard solutions of andrographolide with concentrations of 7.8, 15.625, 31.25, 62.5, 125, and 250 µg/mL were prepared and analyzed to verify the linearity of the resultant calibration curves. Analysis of standard solutions was repeated three times for each concentration and then analyzed using UPLC. Linear least squares regression was used to calculate the slopes, intercepts and correlation coefficients. The acceptance requirement is the value of the correlation coefficients of 0.998 (LoBrutto and Patel, 2007).

2.11. Precision and accuracy

The precision of the analytical method, expressed as the relative standard deviation (%RSD), was determined by analyzing calibration standards prepared at low, medium, and high concentration levels using the standard solution (unspiked sample). Intra-day and inter-day precision were evaluated by preparing three independent calibration standards on different days. Accuracy was assessed by calculating the percentage of relative error between the measured concentration and the expected concentration at low, medium, and high concentration levels for each standard. A limit of ±15% was deemed acceptable for both precision and accuracy (LoBrutto and Patel, 2007)

2.12. Limit of detection (LOD) and limit of quantification (LOQ)

The limits of detection (LODs) and quantification (LOQs) were determined as the minimum concentrations of standard solutions or the lowest spiked concentrations in fortified samples that generated a signal-to-noise ratio of 3 and 10, respectively. The detection and quantification limits were calculated using the following formula (Hwang et al., 2017; Syukri et al., 2015).

$$LOD = 3{,}3 \times \frac{SD}{a}$$

$$LOQ = 10 \times \frac{SD}{a}$$

with:

SD = Standard Deviation

a = The slope of the calibration curve

3. Results and discussion

3.1. Optimization of UPLC-PDA condition

The chromatography system on the UPLC (Ultra Performance Liquid Chromatography) instrument is a modern liquid chromatography system, which has been modified from HPLC. UPLC works at high pressure in the range of 6,000 – 15,000 psi. The smaller particle size (less than 2 m diameter) in UPLC will provide better resolution, speed, and sensitivity compared to HPLC. Smaller particles can increase the pressure to 1000 bar or more which can increase the separation retention factor. Smaller injection volumes are also required in UPLC systems, which results in better efficiency and resolution. In addition, higher column temperatures can also reduce the mobile phase viscosity, diffusion coefficient and high flow rate without significantly reducing efficiency and increasing back pressure on the column (Batool and Menaa, 2020; McShane et al., 2014; Rahman, 2018).

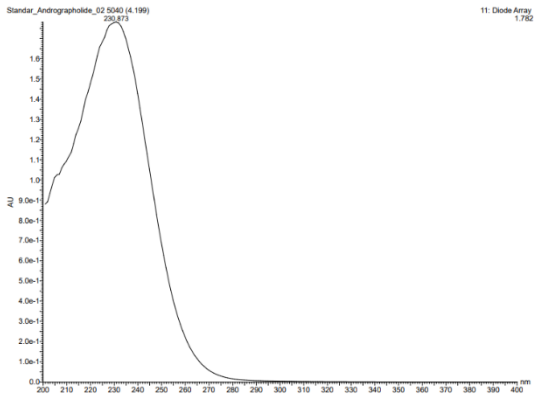

Fig. 1. UV-Vis spectrum of andrographolide solution (500 µg/mL, methanol)

For the analysis, we optimized the UPLC conditions to obtain high separation and resolution of Andrographolide. To enhance chromatographic separation and resolution capacity, 0.1% formic acid (v/v) water and 0.1% formic acid (v/v) CAN were used as mobile solvents with a gradient elution system. The detector was set to 230

nm based on the absorption maximum of andrographolide (Fig. 1). The use of mobile phase at acidic pH by addition of appropriate buffers or acids suppresses the ionization of free silanol groups on silica surface, which reduces ion exchange mechanism of retention. In the acidic mobile phases will also reduce the degree of peak tailing observed and symmetrical peaks when chromatographic basic compounds (Petruczynik, 2012). Various combinations of acetonitril with water or 0.1% formic acid in water and acetonitrile were tried. With an increase in acetonitrile and composition of formic acid (v/v) peak shapes were improved. The UPLC–PDA conditions were optimized by varying the mobile phase, column temperature, and ultraviolet (UV) detection wavelengths (Fig. 2).

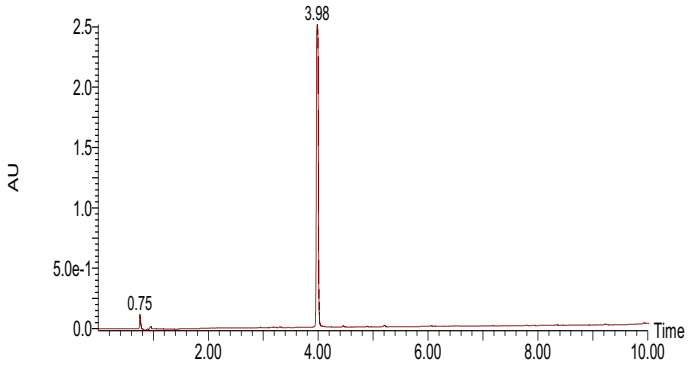

Fig. 2. UPLC- Chromatogram of Andrographolide (500 µg/mL, methanol).

3.2. Method validation

3.2.1. System suitability

The optimized UPLC-PDA method was subjected to a system suitability test. A sample was injected six times in the system and the Retention time (Tr). peak area (AUC). tailing factor (T). and theorical plat number (N) were determined. As shown in Table 1. the RSD values of all these parameters for sennoside B as well were below 2% which indicates all parameters of the proposed UPLC-PDA method satisfy the USP and ICH standards. Therefore. the developed UPLC method is concluded to be suitable and effective for the analysis.

Table 1 System suitability test of the developed method

Parameters	Andrographolide
Retention time (min)	
Mean	3.97
RSD (%)	0.25
Peak area	
Mean (area/µg)	5123.06
RSD (%)	0.94
Tailing factor	
Mean	1.13
RSD (%)	1.38
Theorical plate number	
Mean	252.41
RSD (%)	1.02

3.2.2. Specificity

By comparing the chromatograms of the standard solutions, blank solution (MeOH) and the extract of *A. paniculata*. The study concluded that the andrographolide peak was effectively separated without any interference from other components in the *A. paniculata* extract sample. Specificity was established by comparing the retention times and PDA spectral patterns of the standard solutions, blank solution, and A. paniculata extract sample, which demonstrated that the method accurately identified andrographolide in the test samples. The results of specificity can be seen in Fig. 3.

3.2.3. Linearity, range, LOD, and LOQ

Linearity is the ability of analytical methods that provide a direct response or with the help of a good mathematical transformation, proportional to the concentration of the analyte in the sample. Linearity testing was conducted in triplicate at varying concentrations, and the experiment demonstrated a strong linear relationship (R2 ≥ 0.998) for andrographolide standard solutions in the concentration range of 7.81–250.0 µg/mL. Linear regression analysis of andrographolide yielded the equation y = 41.304x – 71.933. The LOD values of andrograholide was 0.068. The LOQs of andrograholide was 0.205 µg/mL. The results of linearity, LOD and LOQ validation for andrograholide standard can be seen in Table 2.

Table 2. Result of linearity and sensitivity test.

Parameters	Andrographolide
Retention time (min, n=3)	3.98 ± 0.000
Regression equation	y= 41.304x – 71.933
Correlation coefficient (R^2)	0.9999
Linear ranger (µg/mL)	7.81 – 250
LOD (µg/mL)[a]	0.068
LOQ (µg/mL)[b]	0.205

[a]LOD: 3.3 × (standard deviation of the response/slope of calibration curve
[b]LOQ: 10 × (standard deviation of the response/slope of calibration curve)

3.2.4. Precision and accuracy

Accuracy is an analytical method that describes the closeness of the value obtained by the method to the actual concentration value, while the precision describes the closeness of the repeatability of the test sample measurement. Accuracy and precision test were carried out by testing 3 variations of concentration of low, medium and high concentrations. Accuracy and precision observations were carried out intra-day and inter-day with three replications for andrographolide standard concentration. Accuracy is indicated by the percentage value of relative error (%RE), while precision is indicated by the percentage value of relative standard deviation (%RSD). Accuracy and precision requirements are not more than ±15% (EMA, 2011). The results of the validation of intra-day and inter-day accuracy (%RE) and precision (%RSD) are provided in Table 3. Consequently, the UPLC-PDA analytical method proved reliable and reproducible.

Table 3. Result of accuracy (%RE) and precision (%RSD) test of andrographolide.

Concentration (mg/L)	Intra-day (n=3)		Inter-day (n=3)	
	RSD[a] (%)	RE[b] (%)	RSD (%)	RE (%)
200	0.03	2.41	0.78	-1.22
60	0.59	5.21	0.11	3.11
10	0.15	1.07	0.61	1.87

[a] RSD = Relative standard deviation; [b] = Relative error

3.3. Quantitative analysis of andrographolide in *A. paniculata* extract and profile TLC chromatogram

Andrograholide levels were measured using a validated analytical method. The instrument used is UPLC which has good separation due to the smaller column size and higher sensitivity compared to other instruments. Based on the results of the analysis, it shows that the extract of *A. paniculata* extracted using Ultrasound-Assisted Extraction (UAE) contain andrographolide is 4.52 ± 0,06 mg/g (w/w). The calculation results can be seen in the Table 4. Royani et al. (2014), reported that levels of andrographolide produced from 12 regions on the island of Java varied from 0.29 to 4.44% with an average content of 2.19% dry weight. A similar study conducted by Sharma et al., (2013), reported that andrographolide levels using five samples at different plant life cycle stages showed andrographolide levels varying from 0.42% to 2.02%.

Table 4 Result of andrographolide content in the *A. paniculata* Leaf

Sample	Andrographolide content	
	(mg/g±SD)	(%±SD)
A. paniculata leaf	4.52 ± 0.06	0.45 ± 0,00

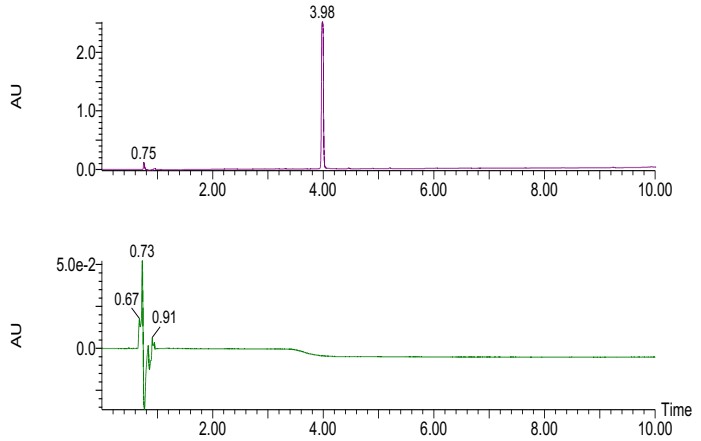

Fig. 2. Overlap chromatogram UPLC for specificity, (a) extract *A. paniculata*; (a) andrographolide standard; (b) blank solution (MeOH).

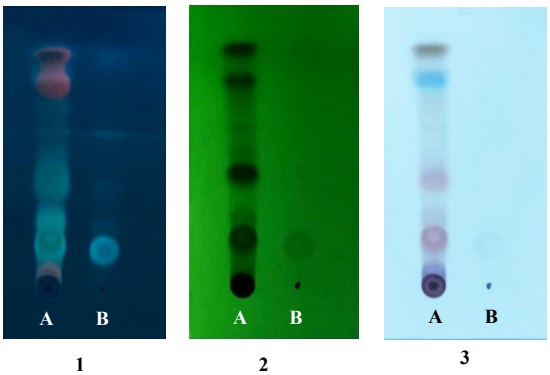

Fig. 4. TLC chromatogram, (a) *A. paniculata* extract; (b) standard andrographolide. The mobile phase used is hexane: ethyl acetate (2:8) and detected using, (1) 254 nm; (2) 366 nm and, (3) spray with sulfuric acid

Thin layer chromatography (TLC) is a cost-effective, sensitive, and fast analytical technique that is utilized for various purposes such as determining the number of components in a mixture, validating the purity and identity of a compound, monitoring the reaction progress, identifying the optimal solvent composition for preparative separations, and analyzing extract or simplisia. The TLC technique is particularly useful for beginners, as it provides detailed instructions and steps for conducting a TLC experiment, complete with illustrations of the necessary instruments and techniques for obtaining and interpreting results (Cai, 2014). In addition, TLC and HPLC profiling was carried out to visually see the presence of andrographolide in the extract based on the chromatogram of each test instrument. Can be seen in the Fig. 4 and Fig. 5. From the results of TLC profiling, it was found that *Andrographis paniculata* contains andrographolide compounds based on the profiling obtained. From the TLC information, the Rf value of andrographolide is 0.18. In addition, profiling using the UPLC instrument showed that the presence of andrographolide compounds was present in these plants. The retention time values were the same between standard and *Andrographis paniculata* extract.

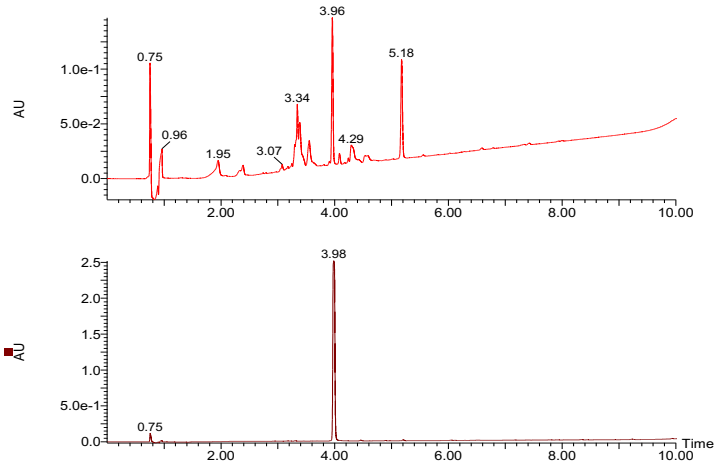

Fig. 3. Overlap chromatogram UPLC: (a) extract *A. paniculata*; (b) andrographolide standard

4. Conclusion

In this study, among the complex mixture of biologically active compound in *Andrographis paniculata*, Andrographolide can be used as an analytical marker compound to determine the quality of plant material of different sources. During crop improvement and drug analysis, a sensitive and accurate analytical method is required for the quantitation of important compound like Andrographolide, which is present in the plant. The UPLC method was validated according to the guidelines for drug development set by ICH (International Council for Harmonisation) and EMA (European Medicines Agency). The UPLC method used a gradient elution system with 0.1% formic acid in water and acetonitrile, which improved the analytical performance. The validation of the method was optimized by assessing various parameters such as specificity, linearity, LOD, LOQ, precision, and accuracy. Quantitative analysis showed that the *A. paniculata* extract contained 4.52±0.06 mg/g of andrographolide. Based on the results obtained, it can be concluded that the proposed methods are dependable and uncomplicated, and have the potential for further improvement. These findings have significant implications for quality control analysis and

standardization of *A. paniculata* extract, as well as for the analysis of andrographolide in other related plant species.

Acknowledgements

This work was internally funded by EBM Scitech

Conflict of interest

The authors declare there is no conflict of interest in this study.

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