ORIGINAL ARTICLE

HPLC-UV Method Validation for the Quantification of Andrographolide and Neoandrographolide in *Andrographis paniculata* Extracts

Subashini Raman[1], Vikneswaran Murugaiyah[1,2], *Thaigarajan Parumasivam[1]

[1] School of Pharmaceutical Sciences, Universiti Sains Malaysia, 11800 Malaysia
[2] Center for Drug Research, Universiti Sains Malaysia, 11800, Penang, Malaysia

ABSTRACT

Introduction: Andrographolide and neoandrographolide are the diterpenoids isolated from *Andrographis paniculata* (Malay: Hempedu bumi). These compounds showed a plethora of biological activities. This study aims to develop and validate a RP-HPLC method for quantifying andrographolide and neoandrographolide in *A. paniculata* extracts. **Methods:** The chromatographic system employed was HPLC Shimadzu L20AD equipped with a Phenomenex-Luna RP-C18 column, while the mobile phase was acetonitrile and water in the ratio of 30:70 v/v at a flow rate 1.0 ml/min. The detection was performed at 210 nm and the retention time of andrographolide and neoandrographolide were 10.5 ± 0.2 min and 26.7 ± 0.5 min, respectively. The total run time was 35 min and the injection volume was 10 µL. **Results:** A linear calibration curve was obtained for both compounds with concentration ranges between 1 and 200 µg/ml. The accuracy was found to be 89.6 % - 113.2 % and the precision was about 0.82 %. The limit of detection (LOD) and limit of quantitation (LOQ) were 4.65 µg/ml and 14.11 µg/ml, respectively for ADG and 4.12 µg/ml and 12.48 µg/ml, respectively for NEO. Conclusions: Hence, this RP-HPLC method is considered eco-friendly due to the minimal usage of organic solvent and rapid due to the relatively shorter duration for the quantification of andrographolide and neoandrographolide.

Keywords: *Andrographis paniculata*; Andrographolide; Neoandrographolide; HPLC validation.

Corresponding Author:
Thaigarajan Parumasivam, PhD
Email: thaigarp@usm.my
Tel: +6016-551 7410

INTRODUCTION

Andrographis paniculata (Burm.f.) Nees which is a well-known Asian medicinal plant of the Acanthaceae family. *A. paniculata* is sometimes referred as "Kalmegh" or "Hempedu bumi"(1). It is a herb that originated from Taiwan, Mainland China, and India. However, it is now widely distributed throughout Asian countries, including Malaysia (2). The herb has a long history of medicinal use in conventional Chinese and Ayurvedic medicine (3,4). It is also used to treat myocardial ischemia and pharyngotonsillitis in modern medicine (1,5,6). It functions as an immune system stimulant and has a number of other beneficial effects, including anti-HIV, anti-cancer, anti-microbial, and anti-inflammatory activities (7,8). World Health Organization (WHO) monograph on Herba Andrographidis reported that *A. paniculata* has activity against the common cold, pharyngotonsillitis, uncomplicated sinusitis, bronchitis, acute diarrhoea, and urinary infections (9).

The major phytoconstituent of the plant are diterpene lactones, including andrographolide (ADG), neoandrographolide (NEO), and deoxyandrographolide. ADG (C20H30O5) is a labdane diterpenoid with a molecular weight of 350.4. While NEO (C26H40O8) is a diterpene glucoside with a molecular weight of 480.6. Both ADG and NEO are insoluble in water and poorly soluble in most organic solvents except methanol, acetonitrile and acetone. Several dosage forms of *A. paniculata*, both in the form of plant extract and pure andrographolide, are presently accessible in the market as finalised herbal products for human consumption. Hence, there is a demand for an eco-friendly technique for the quantification of lead compounds in *A. paniculata* extracts, particularly for the standardisation and quality control of the plant extract's production (10,11). This study aims to develop a less solvent HPLC method for the simultaneous quantification of ADG and NEO in *A. paniculata* extract. The parameters tested are selectivity, linearity, accuracy, precision, limits of detection and quantification.

MATERIALS AND METHODS

Chemical and reagents

ADG and NEO standards were purchased from Toronto Research Chemicals, Canada (TRC). Acetonitrile and methanol were purchased from Merck and distilled water was obtained from Pharmaceutical Technology Lab, USM.

Instruments

High-Performance Liquid Chromatography (Shimadzu LC-20A) equipped with pumps, autosamplers, C-18 reverse phase column (Phenomenex-Luna RP-C18; 250×4.6 mm, 5 µm i.d), photodiode array detector and data processor. A wavelength of 210 nm is chosen to analyse both ADG and NEO.

Preparation of stock solution

10 mg of ADG and NEO was accurately weighed and dissolved in 10 mL of acetonitrile to obtain a stock solution of 1mg/ml. The solution was sonicated for 10 min and working solutions of 200, 100, 50, 25, 10, 5 and 1 µg/ml were prepared by serial dilution.

Method validation

The method was validated following ICH guidelines for selectivity, linearity, accuracy, precision, limits of detection and quantification.

Selectivity, Specificity and System Suitability

The selectivity was determined using the standard solutions with an injection volume of 10 µL and a wavelength (λ) of 210 nm. The mobile phases tested were consisting of methanol:water or acetonitrile:water at various ratios (30:70, 50:50, 60:40, 65:35, 70:30, 90:10) at a flow rate of 1.0 mL/minutes with a maximum pressure of 12 kPa. An optimum mobile phase composition choice was made based on the resolution (Rs > 1.5), tailing factor (< 2), and retention time (12,13).

Linearity & Range

The linearity and calibration curve were evaluated with seven standards (in methanol) containing different concentrations of ADG and NEO (1, 5, 10, 25, 50, 100 and 200 µg/ml). The solutions were sonicated and filtered using 0.22 µm membrane filter before being injected into the HPLC system. The calibration curve was plotted between the area under the curve vs. concentration. In addition, linearity was determined using the regression analysis of the calibration data and the coefficient of variation (r) which is expected to be > 0.999 (14).

Accuracy

To determine the accuracy, known concentrations of ADG and NEO (1, 25, and 200 µg/ml) were prepared in methanol. These solutions were filtered using 0.22 µm membrane filtration subjected to HPLC system using the optimised method. The accuracy was expressed as a percentage recovery in the range of 80-120 % (14,15).

Precision

Precision was determined by analysing the ADG and NEO solutions at three concentrations (1, 25, and 200 µg/ml) injected in triplicates. The ratios of the standard deviation (SD) to the mean were used to determine the relative standard deviation values (RSD), which were then reported as percentages. The precision indicated in terms of relative standard deviation values (RSD) must be within 15% (14,15).

Limits of detection and quantification (LOD and LOQ)

The limits of detection and quantification were determined using ADG and NEO standard solutions ranging from 1.0 – 200 µg/ml. LOD and LOQ were calculated using signal-to-noise (S/N) method in regression model data analysis using Microsoft Excel (16,17).

Intraday and Interday

ADG and NEO solutions at three concentrations of 1, 25, and 200 µg/ml (low, medium, high) were injected in triplicates in the morning, evening and night on the same day, followed by three consecutive days for intraday and interday accuracy and precision.

Sample analysis

A. paniculata ethanolic extract (AP Extract) was dissolved in methanol and, ADG and NEO content were quantified using the developed method.

RESULTS

Method Optimisation

It was found that using the composition of mobile phase, methanol:water (65:35 and 70:30, v/v) and acetonitrile:water (50:50, 60:40 and 90:10, v/v), resulted in a low peak resolution (Rs < 1.5) and high tailing factor (>1.2) for ADG. While acetonitrile:water (30:70, v/v) showed Rs > 1.6 and tailing factor ~1.07. Hence, acetonitrile:water (30:70, v/v) was chosen for further validation. These data are presented in the supplementary data.

Method Validation

The representative chromatogram of the ADG and NEO is shown in Figure 1. The mobile phase of 30:70, v/v acetonitrile:water (Table I) produced good symmetrical peaks for both ADG and NEO. Specificity and system suitability shows that ADG and NEO are stable and reproducible within the same RT using the optimised method as shown in Table I. The retention time of ADG is 10.3 ± 1 mins while 26.2 ± 1 mins for NEO.

Table I : HPLC Conditions

HPLC Conditions	
Column	C_{18} column (Phenomenex-Luna RP-C18)
Size	250×4.6 mm, 5 µm.
Temperature	25°C
Mobile Phase	acetonitrile: water (30:70, v/v)
Flow Rate	1 mL/min.
Injector Volume	10 µL
Retention Time	35 minutes
Wavelength	210 nm (4 nm bandwidth)

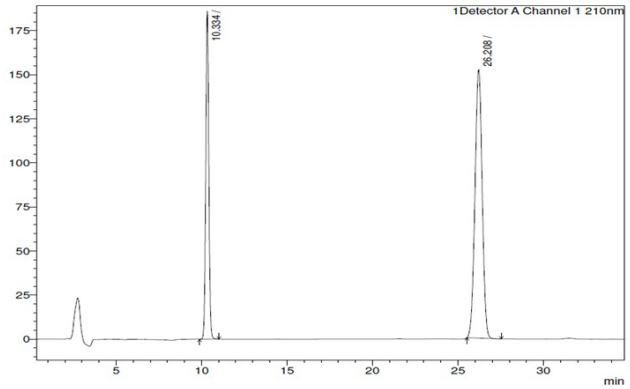

Figure 1 : ADG and NEO chromatogram with the retention time of 10.3 and 26.2 mins, respectively.

AP extract Analysis

Figure 2 shows a sample analysis of AP extract chromatogram for ADG and NEO. ADG and NEO eluted at a retention time of 10.5 and 27.4 mins, respectively.

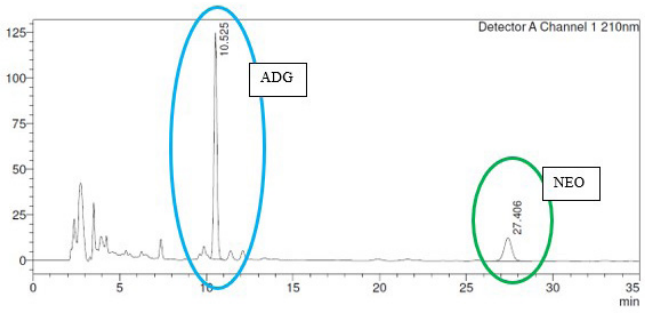

Figure 2 : Chromatogram of *Andrographis paniculata* extract analysis. ADG and NEO showed the retention time of 10.5 and 27.4 mins, respectively.

DISCUSSION

In the present study, we have developed a reverse-phase (RP) HPLC method for the quantification of ADG and NEO in a plant extract. Ultra Performance Liquid Chromatography (UPLC) and liquid chromatography–mass spectrometry (LCMS) are the most commonly used for NEO analysis (18,19). In addition, Zhao et al. (2002) showed that NEO produced better peak shapes using a micellar electrokinetic capillary chromatography (MEKC) as compared to HPLC, which suggests better separation efficiency of the MEKC method compared to HPLC. Similarly, Chandra et al. (2016) developed an UPLC-triple quadrupole/linear ion trap (QqQLIT) –MS/MS method for simultaneous detection of ADG and NEO. However, the present method offers the unique advantage of separating ADG and NEO in a single comprehensive approach using RP-HPLC with UV detection in a relatively shorter duration. The method showed good separation for both ADG and NEO. The method also used only 30% organic solvents as

Table II : Accuracy and Precision of ADG and NEO standards (n=3)

Sample	Day	Concentration (µg/ml)	Mean Calculated Concentration ((µg/ml)	Accuracy (% R.E)	Precision (% R.S.D)
ADG	Intra-day	1	0.91 ± 0.01	-8.57	1.63
		25	24.75 ± 0.09	-1.00	0.35
		200	200.58 ± 1.29	0.29	0.65
	Inter-day	1	0.96 ± 0.01	-3.83	1.04
		25	25.95 ± 0.36	1.94	0.94
		200	198.30 ± 2.01	- 0.85	1.01
NEO	Intra-day	1	0.98 ± 0.01	-2.40	1.43
		25	25.56 ± 0.14	2.25	0.57
		200	197.66 ± 1.26	-1.17	0.64
	Inter-day	1	0.93 ± 0.01	-7.04	1.57
		25	25.26 ± 0.34	1.03	1.34
		200	196.76 ± 1.44	-1.62	0.73

compared to other published methods that used more than 30% organic phase in the mobile phases (10,11,21–31). This method omits the addition of acids in the water phase to ease the mobile phase preparation, yet could separate ADG and NEO simultaneously.

Mobile phase composition of methanol:water (65:35 and 70:30, v/v) and acetonitrile:water (50:50, 60:40 and 90:10, v/v) resulted in an inadequate peak resolution with a high tailing factor, which is in line with Kumar et al. (2014) (32). While good symmetrical peaks for both ADG and NEO were generated by the mobile phase of 30:70, v/v acetonitrile:water (Table I). The RT was also consistent for ADG and NEO when injected at various concentrations, intra-day and inter-day, justifying the reproducibility of the method.

Calibration curves were linear for both analytes of interest (ADG and NEO) over the concentration ranges of 1.0 – 200 µg/ml. Calibration curves were constructed using the concentration versus peak area and analysed by simple regression analysis of y = mX+ C. The linearity data obtained for ADG and NEO are shown in the calibration plot in supplementary Figures 1(a) and 1(b). The regression (r) showed that this method has good sensitivity with % RSD below ≤ 2%.

Three different concentrations of the ADG and NEO were used to assess the precision and accuracy of the measurements. In contrast to accuracy, which is assessed as the percentage of relative error (% R.E), precision was calculated as the relative standard deviation (RSD) over the concentration range (33). A high accuracy and precision scores showed the method is suitable for inter and intra-day analysis.

The LOD and LOQ were found to be 4.65 µg/ml and 14.11 µg/ml, respectively for ADG, and 4.12 µg/ml and 12.48 µg/ml, respectively for NEO. The overall mean recoveries calculated for ADG and NEO were within 95% - 97% which shows good recoveries for all concentrations tested.

The interday and intraday accuracies and precisions were ≤ 2% for both ADG and NEO. The relative standard deviation (RSD %) of precision ranged from 0.35% to 1.63% for ADG and from 0.57 to 1.57% for NEO. US FDA (2000) guidelines stated that accuracy and precision values should not exceed 15% of the relative error (% R.E) and relative standard deviation (% R.S.D) which shows the proposed method has good repeatability and reproducibility (34,35).

ADG and NEO in the extract eluted at a retention time of 10.5 and 27.4 mins, respectively which is similar to the standard solutions. Hence, it can be concluded that the developed HPLC method can be utilised for the quantification of ADG and NEO in AP extracts.

CONCLUSION

An eco-friendly and reproducible RP-HPLC method has been developed and validated to quantify ADG and NEO in AP extract. The method's selectivity, linearity, accuracy, precision, limits of detection, and quantification are all within acceptable limits of ICH guidelines. Hence, this method is suitable for analysing the concentration of ADG and NEO in AP extracts.

ACKNOWLEDGEMENT

The work was supported by Technology Development Fund 1 (TeD1), Ministry of Science, Technology and Innovation (MOSTI) (grant no.: 305.PFARMASI.614401 and TDF03211038) and University Sains Malaysia (USM).

REFERENCES

1. Raman S, Murugaiyah V, Parumasivam T. *Andrographis paniculata* Dosage Forms and Advances in Nanoparticulate Delivery Systems: An Overview. Molecules. 2022 Sep 20;27(19):6164.
2. Hossain MS, Urbi Z, Sule A, Rahman KMH. *Andrographis paniculata* (Burm. f.) Wall. ex Nees: A review of ethnobotany, phytochemistry, and pharmacology. Vol. 2014, Scientific World Journal. Hindawi Publishing Corporation; 2014.
3. Liu XY, Niu X, Feng QJ, Yang XZ, Wang DW, Zhao T, et al. A new biocompatible microemulsion increases extraction yield and bioavailability of *Andrographis paniculata*. Chin J Nat Med. 2016 Sep 1;14(9):683–91.
4. Pawar A, Rajalakshmi S, Mehta P, Shaikh K, Bothiraja C. Strategies for formulation development of andrographolide. Vol. 6, RSC Advances. Royal Society of Chemistry; 2016. p. 69282–300.
5. Tung YT, Chen HL, Tsai HC, Yang SH, Chang YC, Chen CM. Therapeutic Potential of Andrographolide Isolated from the Leaves of *Andrographis paniculata* Nees for Treating Lung Adenocarcinomas. Evidence-Based Complementary and Alternative Medicine. 2013;2013:1–8.
6. Yan Y, Fang LH, Du GH. Andrographolide. In: Natural Small Molecule Drugs from Plants. Singapore: Springer Singapore; 2018. p. 357–62.
7. Hossain S, Urbi Z, Karuniawati H, Mohiuddin RB, Qrimida AM, Allzrag AMM, et al. *Andrographis paniculata* (Burm. f.) wall. ex nees: An updated review of phytochemistry, antimicrobial pharmacology, and clinical safety and efficacy. Vol. 11, Life. MDPI AG; 2021.
8. Dai Y, Chen SR, Chai L, Zhao J, Wang Y, Wang Y. Overview of pharmacological activities of

andrographis paniculata and its major compound andrographolide. Vol. 59, Critical Reviews in Food Science and Nutrition. Taylor and Francis Inc.; 2019. p. S17–29.

9. Guccione C, Oufir M, Piazzini V, Eigenmann DE, Jähne EA, Zabela V, et al. Andrographolide-loaded nanoparticles for brain delivery: Formulation, characterisation and in vitro permeability using hCMEC/D3 cell line. European Journal of Pharmaceutics and Biopharmaceutics. 2017 Oct 1;119:253–63.
10. Sajeeb BK, Kumar U, Halder S, Bachar SC. Identification and Quantification of Andrographolide from *Andrographis paniculata* (Burm. f.) Wall. ex Nees by RP-HPLC Method and Standardization of its Market Preparations.
11. Karioti A, Timoteo P, Bergonzi MC, Bilia AR. A Validated Method for the Quality Control of *Andrographis paniculata* Preparations. Planta Med. 2017 Oct 1;83(14–15):1207–13.
12. Karioti A, Timoteo P, Bergonzi MC, Bilia AR. A Validated Method for the Quality Control of *Andrographis paniculata* Preparations. Planta Med. 2017 Oct 1;83(14–15):1207–13.
13. Sajeeb BK, Kumar U, Halder S, Bachar SC. Identification and Quantification of Andrographolide from *Andrographis paniculata* (Burm. f.) Wall. ex Nees by RP-HPLC Method and Standardization of its Market Preparations.
14. Isadiartuti D, Prihantini IK, Agustina AP, Aryani T, Syahrani A. Development and Validation of an HPLC Method for the Determination of p-methoxycinnamic Acid in Rabbit Plasma [Internet]. 2018. Available from: https://ssrn.com/abstract=3478430
15. Apvma. Guidelines for the Validation of Analytical Methods for Active Constituents, Agricultural and Veterinary Chemical Products [Internet]. 2004. Available from: http://www.apvma.gov.au
16. Saint-Hilaire M, Plumain D, Thomé JP, Adam C, Scholl G, Vedy S, et al. Validation of an HPLC-MS/MS method with QuEChERS extraction using isotopic dilution to analyze chlordecone in human serum. Journal of Chromatography B. 2023 Sep;1229:123894.
17. Rao W, Li L, Zhang C, Zheng J, Fan X, Luan B, et al. Development and validation of a stability-indicating RP-HPLC method for the determination of fifteen impurities in rivaroxaban. J Pharm Biomed Anal. 2023 May;228:115325.
18. Xu F fang, Fu S jun, Gu S pan, Wang Z min, Wang Z zhong, He X, et al. Simultaneous determination of andrographolide, dehydroandrographolide and neoandrographolide in dog plasma by LC–MS/MS and its application to a dog pharmacokinetic study of *Andrographis paniculata* tablet. Journal of Chromatography B. 2015 May;990:125–31.
19. Chandra P, Kannujia R, Pandey R, Shukla S, Bahadur L, Pal M, et al. Rapid quantitative analysis of multi-components in *Andrographis paniculata* using UPLC-QqQLIT-MS/MS: Application to soil sodicity and organic farming. Ind Crops Prod. 2016 May;83:423–30.
20. Zhao J, Yang G, Liu H, Wang D, Song X, Chen Y. Determination of andrographolide, deoxyandrographolide and neoandrographolide in the Chinese herb *Andrographis paniculata* by micellar electrokinetic capillary chromatography. Phytochemical Analysis. 2002 Jul;13(4):222–7.
21. Agarwal R, Sulaiman SA, Mohamed M. Open Label Clinical Trial to Study Adverse Effects and Tolerance to Dry Powder of the Aerial Part of Andrographis Paniculata in Patients Type 2 with Diabetes Mellitus.
22. Burana-Osot J, Phattanawasin P, Sotanaphun U, Sukonpan C, Sriphong L, Kumsum A. Validated ultra-performance chromatographic method for simultaneous determination of andrographolide and its four related diterpenoids in *Andrographis paniculata* capsules [Internet]. Vol. 45. Available from: http://www.tjps.pharm.chula.ac.
23. Banerjee T, Singh A, Kumar S, Dhanani T, Gajbhiye NA, Koley TK, et al. Ovicidal and larvicidal effects of extracts from leaves of *Andrographis paniculata* (Burm. f.) Wall.ex Nees against field isolates of human hookworm (Ancylostoma duodenale). J Ethnopharmacol. 2019 May 10;235:489–500.
24. Jindal S, Awasthi R, Singare D, Kulkarni GT. Isolation, characterization and evaluation of anti-proliferative properties of andrographolide isolated from *Andrographis paniculata* on cultured HaCaT cells. Herba Polonica. 2021 Mar 1;67(1):35–45.
25. Kumar S, Dhanani T, Shah S. Extraction of three bioactive diterpenoids from *andrographis paniculata*: Effect of the extraction techniques on extract composition and quantification of three andrographolides using high-performance liquid chromatography. J Chromatogr Sci. 2014 Oct 1;52(9):1043–50.
26. Da', M., Wikantyasning, E., & Suhendi, A. Validated HPLC Method for Determination of Andrographolide in Mixed Herbal Extract. Int. J. Pharm. Sci. Rev. Res. 2015 Dec; 35(2). https://www.researchgate.net/publication/296549703
27. Ihsan BRP, Hafid AF, Primaharinastiti R, Widyawaruyanti A, Yuwono M. Development and validation of hplc method for determination of andrographolide in raw material and tablet of ethyl acetate fractions of *Andrographis paniculata* . Res J Pharm Technol. 2020;13(9):4291.
28. Sharma S, Sharma YP, Bhardwaj C. HPLC quantification of andrographolide in different parts of *Andrographis paniculata* (Burm.f.) Wall. ex Nees. ~ 168 ~ Journal of Pharmacognosy and Phytochemistry. 2018;7(3):168–71.
29. Sharma M, Sharma A, Tyagi S. ACPI Quantitative HPLC Analysis of Andrographolide in Andrographis Paniculata at Two Different Stages of Life Cycle

of Plant [Internet]. Vol. 2. Available from: www.sadgurupublications.com
30. Du Q, Jerz G, Winterhalter P. Separation of andrographolide and neoandrographolide from the leaves of *Andrographis paniculata* using high-speed counter-current chromatography [Internet]. Vol. 984, Journal of Chromatography A. 2003. Available from: www.elsevier.com/locate/chroma
31. Chandrasekaran CV., Thiyagarajan P, Sundarajan K, Goudar KS, Deepak M, Murali B, et al. Evaluation of the genotoxic potential and acute oral toxicity of standardized extract of *Andrographis paniculata* (KalmColdTM). Food and Chemical Toxicology. 2009 Aug;47(8):1892–902.
32. Kumar S, Dhanani T, Shah S. Extraction of Three Bioactive Diterpenoids from Andrographis paniculata: Effect of the Extraction Techniques on Extract Composition and Quantification of Three Andrographolides Using High-Performance Liquid Chromatography. J Chromatogr Sci. 2014 Oct 1;52(9):1043–50.
33. Batrawi N, Wahdan S, Abualhasan M. Analytical Method Validation of High-Performance Liquid Chromatography and Stability-Indicating Study of Medroxyprogesterone Acetate Intravaginal Sponges. Anal Chem Insights. 2017 Feb 18;12.
34. Le THH, Phung TH, Le DC. Development and Validation of an HPLC Method for Simultaneous Assay of Potassium Guaiacolsulfonate and Sodium Benzoate in Pediatric Oral Powder. J Anal Methods Chem. 2019;2019.
35. Raut R, Shaji J. HPLC method validation for quantification of tetrahydrocurcumin in bulk drug and formulation. Futur J Pharm Sci. 2021 Dec;7(1).
36. Ikayanti R, Hafid AF, Primaharinastiti R, Widyawaruyanti A, Yuwono M. HPTLC method development and validation for the determination of andrographolide in raw material and tablet containing ethyl acetate fraction of Andrographis paniculata. Tropical Journal of Pharmaceutical Research. 2021;20(8):1697–704.