

Harvesting Age and Time Effect of *Andrographis paniculata* Leaf on Its Anti-Inflammatory Activity

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Abstract

In this study, Fourier Transform Infrared (FTIR)-, ¹H Nuclear Magnetic Resonance (NMR)-spectroscopy and multivariate statistical analysis were used for the screening of *in vitro* anti-inflammatory activity on three different germplasm accessions namely 11,341 (P), 11,248 (T) and 11,265 (H) of Malaysian *Andrographis paniculata* (*A. paniculata*) leaf. The anti-inflammatory activity by nitric oxide (NO) inhibition assay in relation to the different harvesting ages and times of *A. paniculata* leaf was determined through comparison by partial least-squares analysis (PLS) using SIMCA-P. The PLS allowed the separation and correlation between the NO assay with the phytochemical present due to the effects of different harvesting ages and times. From the PLS plots, conclusions were drawn with respect to the correlation between *A. paniculata* leaf metabolites with the anti-inflammatory results which showed that 180 days after transplanting (DAT) of morning session for accessions T and H, and evening for P gave the highest anti-inflammatory activity.

Keywords

Andrographis paniculata, Anti-Inflammatory, NO Inhibition, Partial Least-Squares Analysis, FTIR, ¹H NMR

1. Introduction

Andrographis paniculata (Burm. F.) Nees or “Hempedu Bumi” belongs to the family Acanthaceae which is widely grown in tropical areas of Asia such as Malaysia, India, Pakistan and Sri Lanka [1]. *A. paniculata* is a traditional Chinese, Southeast Asian and Indian herb and has been of interest in research due to its wide uses in traditional medicine, for example: as anticancer, anti-inflammatory, anti-allergic, immuno-stimulatory, antithrombotic, hypoglycemic and hepatoprotective agent [2]-[8]. Because of its

vast and interesting biological activities, it is desirable to phytochemically investigate this plant in details in order to better understand the responsible bioactive metabolites.

Recently, several reports on the bioactivities of *A. paniculata* particularly anti-inflammation have been published [9] [10]. However, the influence of *A. paniculata* harvesting ages and times on the anti-inflammatory activity has not been discussed. The optimization of the harvest age and time is crucial to standardize the quality of *A. paniculata* based on the major compounds content, *i.e.* andrographolide as it was reported to be decreasing with the delay in harvesting [11]. Therefore, the anti-inflammatory activity related to NO inhibition of *A. paniculata* leaf at different harvesting ages and times in correlation with its overall biomarkers was determined using metabolomics approach as described herein.

2. Materials and Methods

2.1. Solvents and Chemicals

Deuterated methanol- d_4 ($\text{CH}_3\text{OH}-d_4$), non-deuterated KH_2PO_4 , sodium deuterated oxide (NaOD), trimethylsilyl propionic acid- d_4 sodium salt (TSP), deuterium oxide (D_2O) and methanol were purchased from Merck (Darmstadt, Germany). Liquid nitrogen was supplied by MOX-Linde Sdn. Bhd. (Petaling Jaya, Malaysia). Standard compounds such as andrographolide, neoandrographolide and deoxyandrographolide were previously isolated by Professor Dr. Johnson Stanslas from Pharmacotherapeutics Unit, Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. RAW264.7 cells were purchased from American Type Culture Collection (ATCC, Rockville, USA) under the catalogue number of ATCC® TIB-71TM. Dulbecco's Modified Eagle's Medium (DMEM) with and without phenol red, fetal bovine serum (FBS), penicillin streptomycin (Pen Strep) and TrypLETM Express were purchased from Gibco by Life Technologies Inc. (Eggenstein, Germany). Phosphate buffer saline (PBS) was purchased from Invitrogen by Life Technologies. Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 and interferon-gamma (IFN- γ) were purchased from Sigma Aldrich Co. (St. Louis, MO). DMSO was purchased from Merck (Darmstadt, Germany). PBS and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Invitrogen by Life Technologies Inc. (Eggenstein, Germany) and DMSO was purchased from Merck (Darmstadt, Germany).

2.2. Plant Materials and Cultivation

The *A. paniculata* seeds from three germplasm accessions of Malaysia (Accession 11,265, 11,341 and 11,248 which will be designated as H, P and T, respectively) were collected in July 2011 from Ladang Dua, Universiti Putra Malaysia (UPM). The germination and cultivation procedure in this study was performed according to Yusof *et al.* (2014) [12]. Leaf samples were harvested in January, February and March 2012 assigned as 120, 150 and 180 DAT at two different time sessions (morning: 7:30 - 10:30 am; evening: 2:30 - 5.30 pm). Each sample was quenched with liquid nitrogen and ground into powder form before stored at -80°C prior to drying by freeze-dry system (Labconco, Texas, USA) to

remove the water content.

2.3. FTIR Sample Preparation and Measurement

The FTIR sample preparation and measurement in this study were performed according to Yusof *et al.* (2014) [12]. The freeze-dried leaf powder of *A. paniculata* (150 mg) was extracted by sonication in 5 mL commercial-grade methanol (30 min, 40°C). The extraction step was repeated twice and the total combined supernatant was filtered through filter paper (Whatman, 125 mm) and evaporated using miVac Modular Concentrator series (Genevac Ltd, Ipswich, UK). The crude extracts were then kept at -80°C until IR analysis. The crude samples were freeze-dried again before submitting to IR analysis to prevent absorbance interference caused by the water content. IR spectra were recorded using a Spectrum 100 FTIR spectrometer (PerkinElmer, Waltham, MA, USA) with a single reflectance horizontal ATR cell equipped with a zinc selenide crystal. The analyses were made in absorption mode on a small amount of crude sample deposited on the ATR crystal. The spectra of five biological replicates and pure compounds in three technical replicates were obtained in the frequency range 4000 - 280 cm^{-1} with a resolution of 4 cm^{-1} and a total accumulation of 16 scans.

2.4. NMR Sample Preparation and Measurement

Freeze-dried plant leaf (20 mg) was transferred to a 2.0 mL Eppendorf tube. A volume of 1.5 mL of a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O containing 0.05% trimethylsilylpropionic acid sodium salt (TSP, w/w) and methanol- d_4 (1:1) was added to the plant sample. The extraction procedure in this study was performed according to Kim *et al.* (2010), with a minor modification [13]. The Eppendorf tubes were vortexed for 1 minute at room temperature and ultrasonicated for 20 minutes. This step was followed by centrifuging for 10 minutes at 10,000 rpm to remove the insoluble components from which a 0.75 mL clear supernatant was transferred into the 5 mm NMR tube. ^1H NMR spectra were recorded at 26°C on a 500 MHz Varian INOVA NMR spectrometer operating at a proton NMR frequency of 499.887 MHz. The acquisition time of each ^1H -NMR measurement was 3.53 minutes, which consisted of 64 scans. A presaturation sequence was used to suppress the residual H_2O signal. The resulting spectra were manually phased and baseline was corrected using the Chenomx software (v.5.1, Alberta, Canada) with a consistent setting for all spectra. Spectral intensities were binned by equal width ($\delta 0.04$) corresponding to the region of $\delta 0.50 - 10.00$. The region of $\delta 4.70 - 4.90$ representing water, and $\delta 3.23 - 3.36$ representing residual methanol were excluded. 2D J -resolved was recorded for additional support in the metabolite identification.

2.5. Relative Quantification of Targeted Profiling Data

Andrographolide and neoandrographolide were selected from the ^1H NMR spectra and their concentrations are listed in **Table 1**. Deoxyandrographolide, α -glucose, β -glucose, sucrose, choline and aniline were excluded from the quantification due to their overlapping in the 1D NMR spectra. Relative quantification of the identified metabolites

Table 1. Chemical shift assignment of ¹H NMR signals characteristics of selected metabolites in *A. paniculata* leaf extract and *p* value of one-way ANOVA.

Metabolite	Chemical shift (δ)	<i>p</i> value		
		H	P	T
		120 vs 150 vs 180 DAT	120 vs 150 vs 180 DAT	120 vs 150 vs 180 DAT
Andrographolide (A) (1)	6.91 (m), 4.54 (dd, <i>J</i> = 5.0, 10.0 Hz), 4.09 (d, <i>J</i> = 10.0 Hz), 1.92 (s), 1.32 (d, <i>J</i> = 7.50 Hz), 1.16 (s), 0.72 (s)	>0.05	<0.05	>0.05
Deoxyandrographolide (D) (2)	4.16 (d, <i>J</i> = 6.5 Hz)	ND	ND	ND
Neoandrographolide (N) (3)	7.40 (s), 4.27 (d, <i>J</i> = 10 Hz), 1.00 (s), 0.64 (s)	>0.05	>0.05	>0.05
α-Glucose (a-Glc)	5.20 (d, <i>J</i> = 4.0 Hz)	ND	ND	ND
β-Glucose (b-Glc)	4.60 (d, <i>J</i> = 8.0 Hz)	ND	ND	ND
Sucrose (S)	5.40 (d, <i>J</i> = 3.9 Hz)	ND	ND	ND
Choline (C)	3.20 (s)	ND	ND	ND
Alanine (Ala)	1.48 (d, <i>J</i> = 7.5 Hz)	ND	ND	ND

from the *A. paniculata* was based on the mean peak area of the ¹H NMR signal of interest after binning using Chenomx software. Data was analyzed using the Minitab Statistical Software 14.12.0, © 2004 Minitab Inc. PA 16801-9928, USA. Among the groups, significance test was performed using the one-way ANOVA at 5% significance level.

2.6. Nitric Oxide Assay

The anti-inflammation activity of *A. paniculata* crude extracts was evaluated through Nitric oxide (NO) suppression activity based on Lee's method with some modifications [14]. Each *A. paniculata* crude extract of 2 mg was dissolved in 5 μL of DMSO/995 μL DMEM without phenol red. It was then sonicated for 10 minutes without heating, and centrifuged for 10 minutes at 1000 rpm. The solubility of 50% for the methanol crude extract was determined earlier as to estimate the actual concentration of the prepared crude extracts. From the resulted supernatant, 260 μL was pipetted out into 1040 μL DMEM without phenol red as to minimize the use of DMSO in the sample preparation. This is the stock extract which was further diluted into desired concentrations *via* serial dilutions. Two stock concentrations of 1000 and 10,000 μg/mL were subjected to the two-fold dilution in the screening stage. For the actual assay, the concentrations of *A. paniculata* extracts used were in the range of 100 μg/mL - 1 mg/L in a serial dilution.

DMSO of 0.1% in DMEM without phenol red was utilized as a control vehicle in the serial dilutions in order to maintain the final concentration of 0.1% DMSO in each well. These various concentrations were prepared separately in the aseptic microcentrifuge tubes before being dispensed into the wells.

2.7. Cell Culture and Treatment

The RAW264.7 murine macrophages were cultured in 75 cm² plastic culture flask in DMEM with phenol red containing HEPES, L-glutamine supplemented with 10% FBS and 1% penicillin/streptomycin in 5% CO₂ atmosphere at 37°C. After the cells have

reached the confluency of 80% - 90%, they were detached from the flask wall by rinsing off the flask with 5 ml of PBS solution followed by 2 ml of TrypLE™ Express before incubated for 10 minutes. DMEM with phenol red of 4 mL was used to rinse out the detached cells which then centrifuged at 3000 rpm, 4°C for 10 minutes by using Rotina 35R (Andreas Hettich GmbH & Co., Tuttlingen, Germany). The supernatant was then removed and the cells were re-suspended with fresh DMEM containing HEPES, and L-glutamine with 5% FBS but without phenol red. The cells were counted by using a standard trypan blue cell counting technique.

The cell concentration was adjusted to 1×10^5 cells/mL in the same medium (DMEM without phenol red) before seeded in a 96-well flat bottom tissue culture plates at initial seeding densities of 5×10^4 cells/50 μ L per well. The ready plates with seeded cells were incubated at 37°C (5% CO₂ atmosphere) overnight to allow anchoring of cells to the wells.

The treatment of the cells was done by replenishing the medium in each well with 50 μ L DMEM without phenol red containing triggering agent of 1 ng/mL recombinant murine IFN- γ and 1 μ g/mL LPS. For each dilution of the plant extracts, 50 μ L was added into the appropriate wells in four replicates. The assay plate was incubated for 17 hours at 37°C in 5% CO₂ atmosphere. There were five kinds of controls prepared in duplicates for each plate. The controls were media only without cells (blank control), cells in media only, cells in media containing triggering agent plus 0.1% DMSO (negative control), cells in media containing triggering agent only, and cells containing triggering agents plus curcumin (positive control).

2.8. Nitrite Determination

Nitrite concentration was determined by Griess assay through the reaction of 50 μ L of cell culture supernatant and 50 μ L of Griess reagent (1% sulphanilamide and 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid) at room temperature. The optical density was measured at 550 nm after 5 minutes of incubation at room temperature by Spectramax Plus (Molecular Devices) UV/Vis microplate reader. The accumulated NO release was determined spectrophotometrically by the total nitrite in the medium. The nitrite concentration in the samples was determined by regression analysis using serial dilutions of sodium nitrite as a standard. The percentage inhibition was calculated based on the ability to inhibit nitrite below the level produced by cells cultured in the presence of triggering agents and DMSO, which was considered as 0% inhibition.

2.9. MTT Cell Viability Assay

In order to avoid any false positive result of NO inhibition, the treated cells were also submitted for MTT assay as to measure any cytotoxic effect on the cells by using the Heras's method [15]. Supernatant in all wells was removed followed by the addition of 100 μ L DMEM with phenol red. Subsequently, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to each well and the plate

was incubated in 5% CO₂ atmosphere at 37°C for 4 h. The mixture of culture media and MTT in all wells were discarded and the purplish formazan crystals formed were dissolved in DMSO and further incubated for 5 minutes. The absorbance of colour intensity was then measured at 570 nm at the room temperature wherein the absorbance of formazan in the control of untreated cells was taken as 100% viability.

2.10. Statistical Analysis

Data were analyzed by using GraphPad Prism software version 5.01 (GraphPad Software Inc., San Diego, USA). The graph of half maximum inhibitory concentration (IC₅₀) for each sample was obtained from logarithm graph plot. In which each graph showed the goodness of fit, $R^2 > 0.95$ and within the 95% of the confidence interval with p -values < 0.05 were considered significant. All results were expressed as the mean \pm S.E.M of n , where n represented the number of wells replication. Differences between the mean values for different groups were analyzed by Analysis of variance (ANOVA) using SPSS 16.0 (Statistical Package for the Social Sciences).

2.11. Multivariate Data Analysis

Multivariate data analysis by partial least-squares analysis (PLS) was performed with the SIMCA 13.0 software using scaling based on Variance.

3. Results and Discussion

3.1. ¹H NMR Spectra and Metabolite Identification

In the present work, metabolomics analysis was applied to discriminate the metabolite variation among the three accessions of P, T and H. Metabolites from this plant including the major andrographolide, deoxyandrographolide, neoandrographolide and carbohydrates (α -glucose, β -glucose and sucrose) as well as amino acids (alanine and choline) were successfully identified. **Figure 1** shows the ¹H-NMR spectra of *A. paniculata* leaf extracts displaying both the qualitative and quantitative variations of some metabolites. The compounds were identified based on the 2D J -resolved (**Figure 2**) and comparison with the reported NMR signals of those isolated metabolites from this plant. Other compounds were assigned by comparison with the NMR spectra of the reference compounds measured under the same conditions as extracts. All of the spectra showed signals in the aliphatic (δ 0.5 - 3.0), aromatic (δ 5.5 - 8.0) and carbohydrate region (δ 3.0 - 5.5).

Table 1 lists the characterized metabolites which could be recognized by signals shown in those three main regions. The peaks in at δ 0.64 and δ 1.00 were typical signals of the neoandrographolide aliphatic protons, whereas andrographolide signals were detectable at δ 0.72, 1.16, 1.32 and 1.92. Some amino acids namely alanine and choline were also observed in the aliphatic region at 1.48 and 3.20, respectively. Alpha-glucose, β -glucose and sucrose were ascribed based on the signals displayed in the carbohydrate region of δ 3.0 - 5.5. Other andrographolide signals were observed at δ 4.09 and 4.54. As for deoxyandrographolide, the signal was detectable at δ 4.16. Andrographolide and

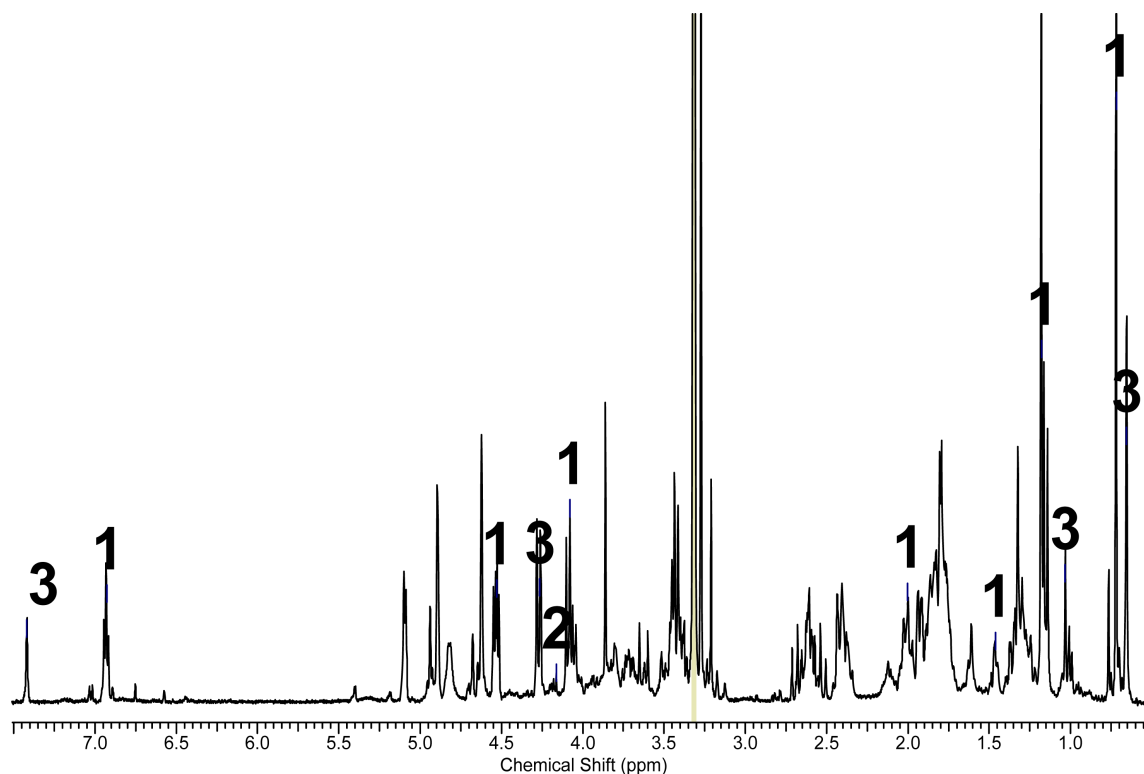


Figure 1. ^1H NMR spectra of the *A. paniculata* leaves extract: expanded range δ 0.50 - 7.50. The observed signal as follows: 1—andrographolide; 2—deoxyandrographolide and 3—neoandrographolide.

neoandrographolide signals were also observed in the aromatic region of δ 6.91 and 7.40 respectively.

3.2. Relative Quantification

Relative quantification of the identified major compounds which are andrographolide and neoandrographolide in *A. paniculata* extracts was based on the mean peak area of the ^1H NMR signals from the three different accessions (P, T and H), which were harvested at three different ages (120, 150 & 180 DAT) and two different time sessions of morning and evening. *A. paniculata* accessions from different ages were able to be differentiated by the intensities of andrographolide (binned at 0.74 ppm) and deoxyandrographolide (binned at 0.62 ppm). The significance ($p < 0.05$) difference between the samples at different age for each accession was conducted by One-Way ANOVA using MINITAB 14. Morning and evening session data for 120, 150 & 180 DAT of three different accessions (P, T and H) were combined in this relative quantification analysis.

The concentration of andrographolide at 180 DAT of P accession was significantly higher ($p < 0.05$) as compared to 120 and 150 DAT as shown in **Figure 3(a)**. Similarly, T accession of the harvesting age at 180 DAT showed the highest concentration of andrographolide (**Figure 3(b)**), but with no significant difference when compared to 120 and 150 DAT. The highest concentration of andrographolide for H accession was observed at 150 DAT instead of 180 DAT with insignificant difference between the three

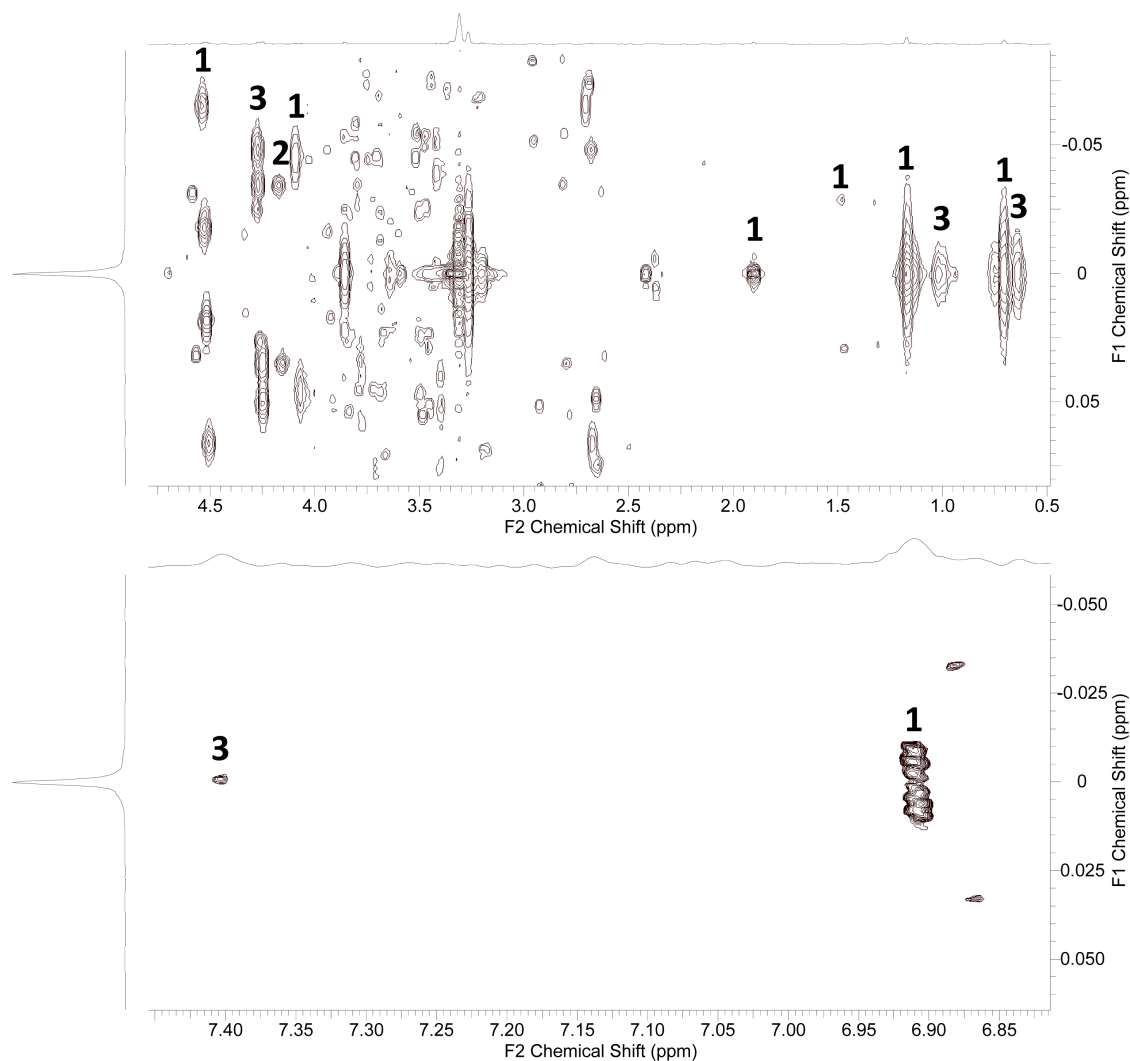


Figure 2. ^1H - ^1H J -resolved spectra of the *Andrographis paniculata* extract in the region δ 0.50 to 7.45. The observed signal as follows: 1—andrographolide; 2—deoxyandrographolide; 3—neoandrographolide.

DATs (Figure 3(c)) (Table 1). The concentration of neoandrographolide as in Figures 3(a)-(c) was observed to be more abundant in 180 DAT harvesting age for H and T accessions, whereas 150 DAT harvesting age for P accession. However, the p value exhibited no significance between all DATs for each accession (Table 1).

3.3. Anti-Inflammatory Activity of *A. paniculata*

Investigation on the in vitro anti-inflammatory effect of the *A. paniculata* accessions methanol crude extracts was carried out via nitric oxide assay. *A. paniculata* has been used traditionally for inflammation since ages [16]. Thus, based on the claims, this is an attempt made in identifying the possible metabolite changes due to the harvesting time and age variations in relation to the anti-inflammation activity. The correlation of the quantitative IC_{50} values from NO assay with the data of FT-IR and FT-NMR has paved the way in identifying the possible biomarkers.

Since the *A. paniculata* leaf extracts have shown promising results at the screening stage, the IC_{50} values were then further determined as summarized in **Table 2** and **Figure 4**. The potential of the *A. paniculata* crude extracts as anti-inflammatory agents

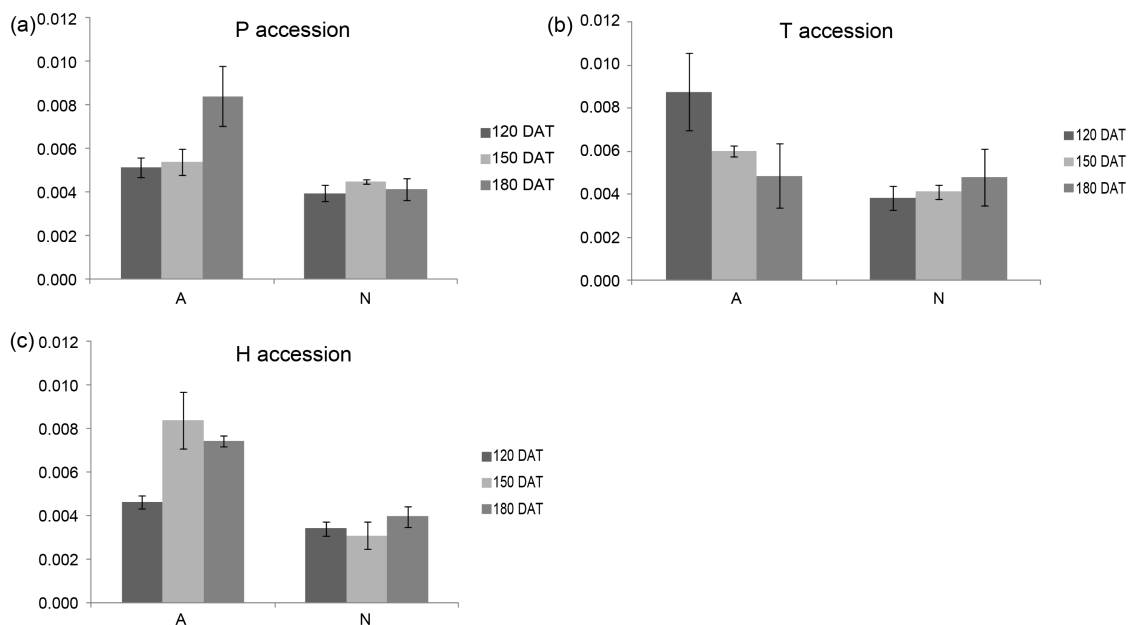


Figure 3. Relative quantification of identified compounds in *A. paniculata* leaves extract (andrographolide (A) and neoandrographolide (N)) based on the mean peak area of the 1H NMR signals from three extractions associated with the identified compounds. Chemical shifts (δH) used for the relative quantification are andrographolide at 0.74 and neoandrographolide at 0.62. Y-axis expresses the relative peak area of corresponding signal to that of internal standard (TSP): (a) P accession (b) T accession (c) H accession.

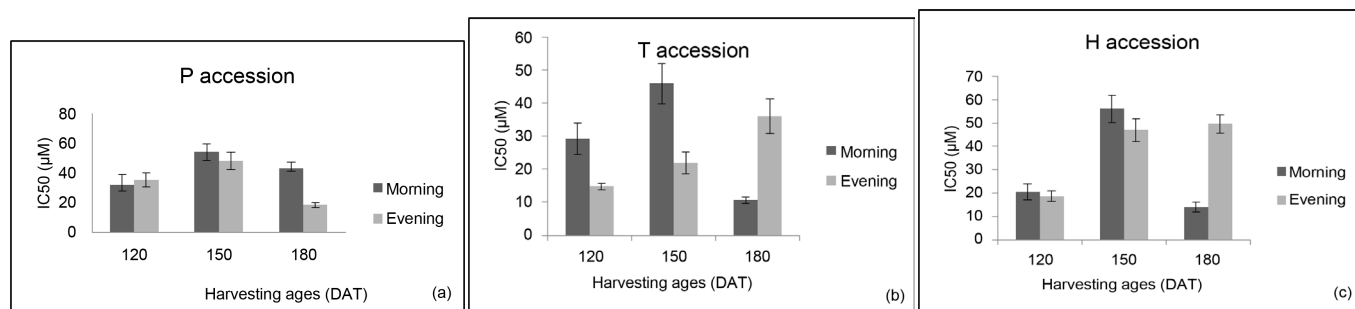


Figure 4. The IC_{50} for each accession of *A. paniculata* leaf: (a) P accession (b) T accession (c) H accession.

Table 2. The IC_{50} values of all accessions for NO assay.

Age	120 DAT			150 DAT			180 DAT		
	P	T	H	P	T	H	P	T	H
Morning									
$IC_{50}/\mu M$	32.35 \pm 6.46	29.21 \pm 4.73	20.47 \pm 3.51	54.56 \pm 4.85	45.83 \pm 6.16	56.20 \pm 5.84	42.94 \pm 4.65	10.61 \pm 0.97	13.99 \pm 2.14
Evening									
$IC_{50}/\mu M$	35.40 \pm 4.60	14.74 \pm 1.04	18.71 \pm 2.35	48.02 \pm 5.88	21.95 \pm 3.27	47.00 \pm 5.02	18.51 \pm 1.62	35.98 \pm 5.24	49.60 \pm 4.02

Standard: IC_{50} curcumin = 14.69 μM ; P = Pasir Puteh, T = Tanjung Ipoh, H = Harapan.

were assessed from the inhibition of the NO production without the induction of cell death. Thus, another test was carried out in order to determine the cell viability of the induced macrophages.

It could be observed that all the three accessions yielded the highest NO activity at 180 DAT harvesting age of morning session for T and H, and evening session for P with IC_{50} values of 13.99, 10.61 and 18.51 $\mu\text{g}/\text{mL}$, respectively. In relation to the NO inhibition, P and H accessions gave a significant difference between 120 and 150 DAT, but no significant difference between the morning and evening harvesting times. However, T accession exhibited a significant variation between morning and evening harvesting time for each of the harvesting age.

Generally, the small IC_{50} value is the lowest concentration that could inhibit the production of NO by at least 50%. However, the statistical computation only recognizes big values in representing a significant activity. Thus, prior to any MVDA computation step, the IC_{50} values were converted into their antiradical scavenging activity ($1/IC_{50}$) readings in order to able the statistically translation of the activity.

3.4. Correlation between Anti-Inflammatory and Metabolite Variation Using FTIR Spectroscopy

PLS was applied using a validation model with a degree of over fit between the variables and the responses [17]. PLS was performed to obtain a more precise view on the correlation between the NO assay and the phytochemical present with the effect of harvesting age variations of 120, 150 and 180 DAT. This approach was implemented because PLS has greater success in linking NO assay with the phytochemicals and in providing models for prediction of the highest bioactivity among the different harvesting ages [17] [18].

Three clusters were obtained from the PLS bi-plot that combined the score and loading plot of the FTIR data (Figure 5). A differentiation analysis of the metabolites between the 180 DAT harvesting age samples from the other two different ages of the

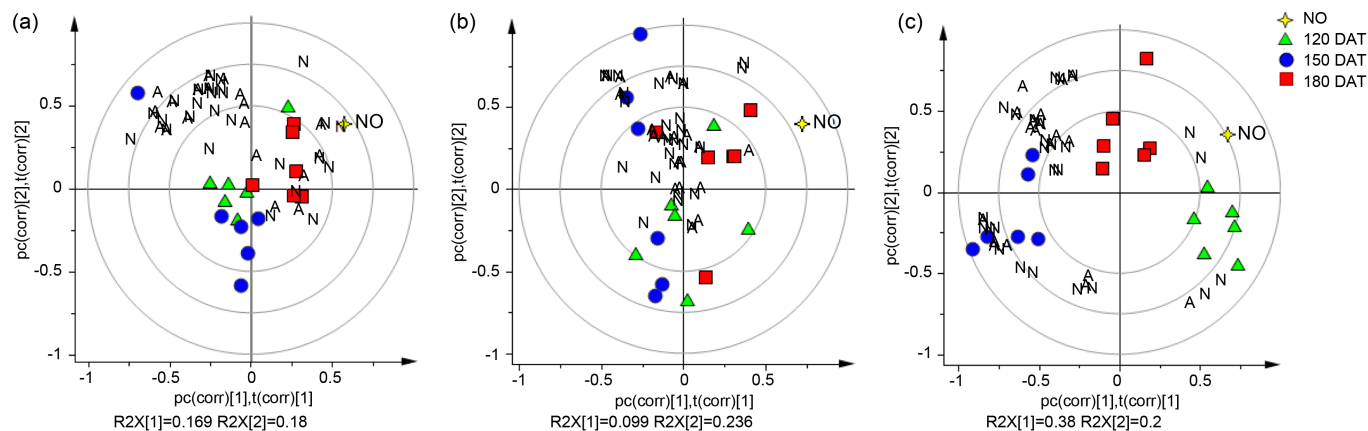


Figure 5. The bi-plot of FTIR data obtained from PLS describing the variation between harvesting ages at 120, 150 and 180 DAT of different accession of *A. paniculata*: (a) P accession (b) T accession (c) H accession. (Abbreviations: A, andrographolide; D, deoxyandrographolide; N, neoandrographolide).

three *A. paniculata* accessions (P, T and H) correlated to NO assay was accomplished (Figure 5). Among the three accessions, P showed that 180 DAT harvesting age was strongly correlated with the NO inhibition activity (Figure 5(a)).

The other two harvesting ages were projected to the negative side of the plot by PC1 which was opposite of the NO. The peak signals were characterized as those belonging to the major compounds of andrographolide and neoandrographolide. The same data distribution was observed over the ellipse for the T accession of different harvesting ages model (Figure 5(b)). The closest harvesting age to the NO inhibitory property was 180 DAT. This group was projected nearer to the NO in the positive quadrant of both PC1 and PC2.

There was a different observation for H accession, whereby all the three harvesting ages were well-separated in groups (Figure 5(c)). The 180 DAT was well discriminated from the other two harvesting ages in the positive side of PC2 which gave 180 DAT as the nearest to having NO inhibition property.

3.5. Correlation between Anti-Inflammatory and Metabolite Variation Using NMR Spectroscopy

A. paniculata samples were analyzed with NMR spectrometer to support the data obtained from FTIR analysis. The PLS loading bi-plot data gave 180 DAT of P and T accessions as well separated from 120 and 150 DAT by PC1 (Figure 6(a) and Figure 6(b)), whereby the 180 DAT of P and T were strongly correlated to NO activity. The characteristic of 180 DAT was observed to be having higher level of the major compounds of andrographolide, neoandrographolide and deoxyandrographolide which could be correlated to the NO inhibition property. This is in agreement with the obtained anti-inflammatory activity data in which exhibited that the 180 DAT of P and T accessions gave the lowest IC₅₀ value for NO. Other metabolites that contributed to the clustering were carbohydrates and amino acids which are shown in the loading bi-plot of Figure 6(a) and Figure 6(b).

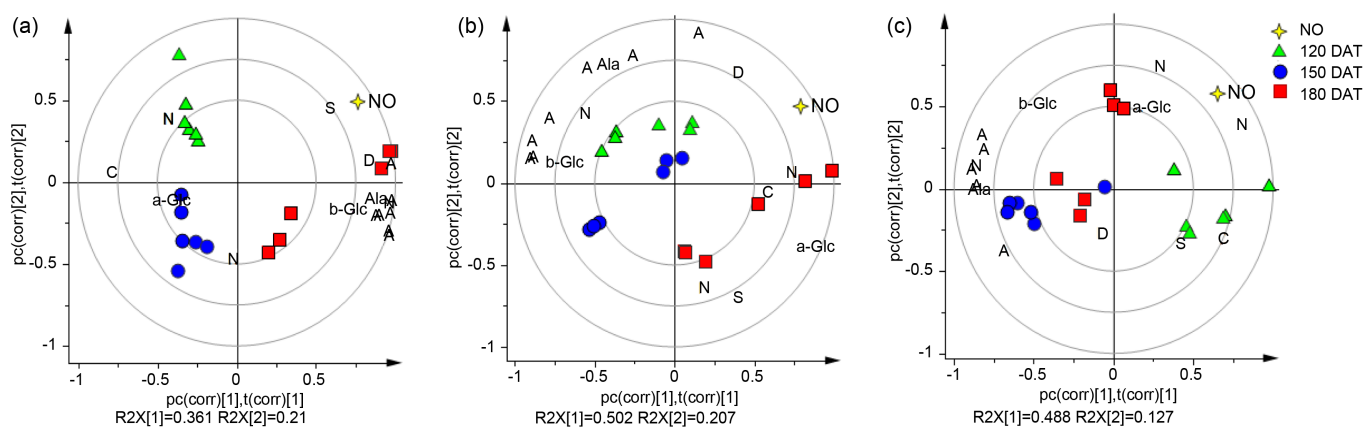


Figure 6. The bi-plot of NMR data obtained from PLS describing the variation between harvesting ages at 120, 150 and 180 DAT of different accession of *A. paniculata*: (a) P accession (b) T accession (c) H accession (*Abbreviations*: A, andrographolide; D, deoxyandrographolide; N, neoandrographolide; a-Glc, α -glucose; B-Glc, β -glucose; S, sucrose; Ala, alanine; C, choline; NO, nitric oxide).

H accession gave a different observation wherein both 120 and 180 DAT were close to NO assay compared to 150 DAT as shown in **Figure 6(c)**. Even though both of the DATs of 120 and 180 situated close to the NO activity, the clustering of these two could be established as 120 DAT was closest to NO by PC1 whereas 180 DAT by PC2 with 180 DAT having the highest NO inhibition. Metabolites that contributed to this separation were andrographolide, neoandrographolide and carbohydrates with higher content of neoandrographolide and amino acids in 120 DAT.

The correlation between anti-inflammatory and metabolite variations among the three different harvesting ages of P, T and H accession using NMR spectroscopy in this study is agreeing with the data obtained from the FTIR analysis. Both of the spectroscopic data are in congruent when correlated to the NO inhibition activity.

3.6. Permutation Test

PLS model validation was performed by checking the Q^2 and R^2 accumulative means after cross validation and permutation test with 100 permutations (**Figure 7**). According to Eriksson *et al.* (2006), the permutation test will generate Q^2 value higher than 0.5, R^2 value must always be larger than the value of Q^2 for a good model [19]. Thus, with

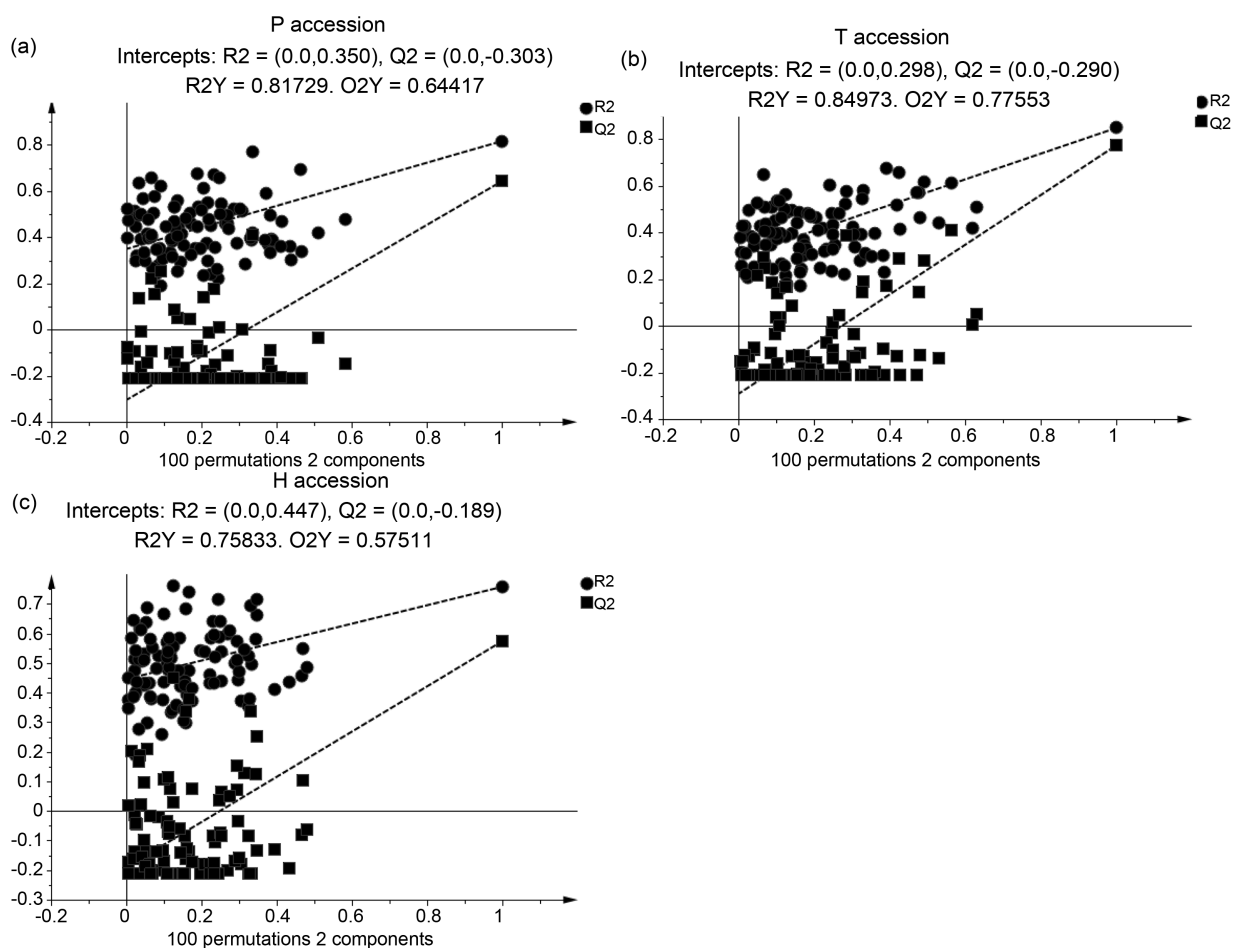


Figure 7. The PLS validation for each harvesting age model for: (a) P accession; (b) T accession and (c) H accession.

the higher significance and lower variation of the NMR-adopted-permutation has helped each model to be validated as a good model. In the current study, the Q^2 and R^2 values were higher than 0.5 (P accession: $R^2 Y = 0.75833$, $Q^2 Y = 0.71733$; T accession: $R^2 Y = 0.84973$, $Q^2 Y = 0.77553$; H accession: $R^2 Y = 0.75833$, $Q^2 Y = 0.57511$) which suggested that all of the models meet the criteria of the validation and prediction performances.

4. Conclusion

Utilizing the FTIR and NMR-metabolomics approach has provided a rapid way to study the metabolites variation of *A. paniculata* in relationship to its different harvesting ages & time with a biological activity of anti-inflammation. The PLS allowed the separation and correlation between the NO assay with the phytochemical present due to the effects of different harvesting ages and times. P (11,341), T (11,248) and H (11,265) accessions exhibited 180 DAT as the best harvesting age possessing the highest anti-inflammatory activity. The accessions at this age also contained higher level of the major compounds which could be attributed to the anti-inflammatory capacity.

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