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HPLC-FTIR spectroscopy combined with multivariate calibration for analysis of Andrographolide in *Andrographis paniculata* extract

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Andrographis paniculata, FTIR spectra, HPLC, PLSR, multivariate calibration.

ABSTRACT

Andrographis paniculata, known as *Sambiloto* in Indonesia, has been reported to have pharmacological activities with the main active constituent being andrographolide (ANDR). The present study highlighted the research for determining the levels of ANDR by correlating the absorbances of Fourier transform infrared (FTIR) spectra with ANDR contents as quantitatively analyzed by reference method of High-performance liquid chromatography (HPLC) using multivariate calibrations. *Andrographis paniculata* herbs from several regions were powdered. The powdered samples were measured in terms of FTIR spectra. Besides, a weighted sample was subjected to the extraction procedure and measured using HPLC. Data obtained are subjected to multivariate calibrations. The result indicated that the method was useful to evaluate ANDR content in *A. paniculata* herb. Partial least square regression (PLSR) using FTIR spectra in the form of a second derivative at the wavenumber regions of 3,700–665 cm^{-1} was finally preferred for the quantitative analysis of ANDR with Coefficient of determination (R^2) values of 0.9997 in the calibration model and 0.9765 in the validation models. The values of Root mean square error of calibration (RMSEC) and Root mean square error of prediction (RMSEP) obtained were 0.005 and 0.055, respectively. Due to its capability of providing a high value of R^2 and low values of RMSEC and RMSEP, the application of PLSR using the variable of FTIR spectra at selected conditions could be an effective alternative method for quantitative analysis of ANDR.

INTRODUCTION

Andrographolide (ANDR), having the chemical structure as shown in Figure 1, is a member of diterpenoid compounds, mainly isolated from *A. paniculata* belonging to the Acanthaceae family. This plant is known as the "King of Bitters." In Indonesia, *A. paniculata* is known as "*Sambiloto*," one of the medicinal plants extensively studied because of some beneficial health effects (Akowuah *et al*., 2009). In traditional medicine, especially in Asian countries, *Sambiloto* is widely used to treat fever, cold, laryngitis, and infections. The extracts and fractions of Sambiloto containing ANDR have been evaluated for the biological activities including antioxidant (Akowuah *et al*., 2008), hepatoprotector from cell death induced by hydrogen peroxide (Mittal *et al*., 2016), carbon tetrachloride (Chen *et al*., 2014), inducer of glutathione S-transferase pi class (Lu *et al*., 2011), inhibitor of inflammatory responses in lipopolysaccaride-stimulated macrophages (Kim *et al*., 2019), and to have antidiabetic activities (Xu *et al*., 2012). These activities are correlated with phytochemical contents present in *A. paniculata*, mainly ANDR; therefore, analytical methods

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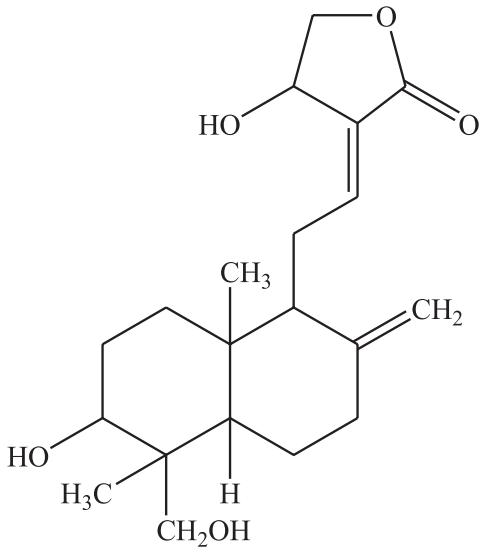

Figure 1. The chemical structure of andrographolide (ANDR).

capable of a fast and reliable technique for the determination of ANDR are continuously developed, validated, and applied in any type of sample matrices.

Several methods have been applied for quantitative analysis of ANDR, and the most reported ones are chromatographic-based methods. High-performance liquid chromatography (HPLC) using detector UV 224 nm has been used for purity analysis of ANDR and ANDR quantification in bulk materials (Indrati *et al*., 2018) and HPLC using detector UV 210 nm has been used for the analysis of ANDR in methanol extract of *A. paniculata*. Liquid chromatography-mass spectrometry has also been widely used for the analysis of ANDR in extracts and biological fluids (Gu *et al*., 2007; Sajeeb *et al*., 2015; Xu *et al*., 2009; Zhang and Fan, 2012). The other methods used for ANDR quantification are thin layer chromatography (Akowuah *et al*., 2006), electrokinetic chromatographic (MEEKC) method (Yanfang *et al*., 2006), and proton NMR-spectroscopy (Yang *et al*., 2012). These methods involve sophisticated instruments, complex sample preparation, and skillful analyst; therefore, a reliable method offering accurate and precise results based on Fourier transform infrared (FTIR) spectra could be developed as an alternative method for the determination of ANDR.

FTIR spectra were reported for characterization of vibrational properties of ANDR extracted from *A. paniculata* (Singh *et al*., 2006) and for confirmation and qualitative analysis of ANDR. FTIR spectroscopy has been successfully used for the analysis of total lactones in dried and powdered *A. paniculata* (Shivali *et al*., 2012). To the best of our knowledge, FTIR spectra in conjunction with chemometrics of multivariate analysis for quantitative analysis of ANDR have not been reported as yet (Indrati *et al*., 2018). Therefore, in the present research, FTIR spectra assisted with Partial least square regression (PLSR) was used for the prediction of ANDR. The levels of ANDR quantified by HPLC were used as actual values to be correlated with predicted values obtained from FTIR spectra with the aid of multivariate calibration.

MATERIALS AND METHODS

Materials

The samples of *A. paniculata* herbs (15 samples) were obtained from several regions in Daerah Istimewa Yogyakarta, West Java, and Central Java (Bantul, Sleman, Kulon Progo, Semarang, Boyolali, and Bogor), Indonesia. The plant identification was carried out in the Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, UGM, Yogyakarta. The reference standards of ANDR and methanol HPLC grade were purchased from Merck (Darmstadt, Germany). Water for injection was bought from Ikapharmindo (Indonesia). The chemicals used for the analysis were of a pro analytical grade.

Samples' extraction

The herbs of *A. paniculata* were cleaned and chopped into pieces. The chopped herbs were dried in a conventional oven for 24 hours. The dried herbs were grounded into a powder. The extraction method used was maceration by weighing 5 g of powdered samples from each region using an analytical balance with a sensitivity of 0.1 mg (Mettler Toledo). The powders were macerated with 50 ml of absolute ethanol pro analytical grade for 24 hours. Filtration was carried out to obtain a liquid extract and then ethanol was added to a volumetric flask of 50.0 ml. This sample solution was analyzed using HPLC.

Analysis of ANDR using HPLC

The reference standards of ANDR were dissolved in methanol HPLC grade to get stock solution with a concentration of 1,000 µg/ml. A series of working solutions in certain concentration ranges (25, 50, 75, 100, and 125 µg/ml) were also prepared from the stock solution. Sample solutions were prepared by transferring 1 ml sample extract into a volumetric flask of 10 ml, filled until 10 mL with ethanol and filtered with 0.45 µm filter before being subjected for injection into LC chromatograph. HPLC method for the analysis of reference standards and sample solutions was carried out according to Syukri *et al*. (2016). HPLC analysis of ANDR was carried out using chromatograph Shimadzu LC-20AD (Kyoto, Japan) equipped with binary gradient pump using injection valve of Rheodyne 7725i with 20 µl loop. HPLC separation was carried out on Cosmosil C_{18} column (250 × 4.6 mm, 5 µm) using a mobile phase of methanol and water (6 : 4 *v/v*) and delivered isocratically at a flow rate of 0.8 ml/minutes. The injection volume and wavelength of the wavelength detector were 20 µl and 229 nm.

FTIR spectra measurement

The measurement of FTIR spectra was carried out according to Irnawati *et al*. (2020b). The powdered *A. paniculata* samples were placed on a Smart iTR™ attenuated total reflectance at a mid-infrared region of 4,000–650 cm^{-1}, recorded for 32 scans at a resolution of 8 cm^{-1}.

Data analysis

The multivariate calibrations were carried out through chemometric software of TQ Analyst® software version 9 (Thermo Fisher Scientific, Inc., Waltham, MA). The multivariate

calibrations used were PLSR and principal component regression (PCR). HPLC data were used as the actual values and FTIR data were used as a predicted value. The selection of wavenumber regions is based on its capability of giving high coefficient of determination (R^2) and low values of errors, either Root mean square error of calibration (RMSEC) or Root mean square error of prediction (RMSEP).

RESULTS AND DISCUSSION

In the present research, FTIR spectra combined with multivariate calibrations were used for the quantification of ANDR in herbs of *A. paniculata*. As its property as fingerprint technique, FTIR spectra could be used for selecting specific peaks corresponding to target analytes (ANDR). But, FTIR spectra used as tools for the analysis of ANDR in herbs are nonstandard methods; therefore, the actual values of analytes must be determined using a reference method, namely, HPLC. Quantification of ANDR using HPLC was carried out using external calibration by preparing the linearity curve correlating between concentrations of ANDR (*x*-axis) and peak area or area under curve (*y*-axis). The linearity was obtained from five concentrations of standard solutions (25, 50, 75, 100, and 125 µg/ml). From the calibration plot in Figure 2, the (R^2) value for ANDR was 0.99998, indicating a good linearity with the following equation: $y = 50{,}513.92 \times -16{,}180.4$.

HPLC, a reference method for analysis of analyte of interest, was utilized for the quantitative estimation of ANDR. Standard and samples showed similar retention time values. Figure 3 shows the HPLC chromatogram, either in ANDR obtained from Sigma-Aldrich (at a concentration of 75 µg/ml) with retention time 7.140 minutes or in ethanolic extract of *A. paniculata* (AP3) with the retention time of 7.121 minutes (data were not shown). An analyte can be characterized by its retention time, which is not affected by the quantity of injected samples. Table 1 shows the concentrations of analyte (ANDR) in some samples of ethanolic extracts of the *A. paniculata* herb in some regions. ANDR contents in the analyzed extracts were diverse, mainly due to the differences in season of cultivation, region, age, and time of harvesting (Hossain *et al.*, 2014). The concentrations of ANDR were used as actual values to be correlated with ANDR contents predicted by the FTIR method facilitated with two multivariate calibrations of PCR and PLSR.

Figure 4 shows the FTIR spectra of dried powder of *A. paniculata* herb from different regions. The main component present in *A. paniculata* is ANDR. The peak and shoulders shown have originated from the functional groups'' absorption present in the evaluated samples. From the analysis, the FTIR spectra showed similar peaks, which can be interpreted as a similar profile in chemical components. The differences in peak intensities caused by different levels of chemical contents could be seen in the dried powders. The peak at (a) 3,286 cm^{-1} may be due to the presence of stretching vibration of the O-H bond. The peaks at (b) 2,919 and (c) 2,851 cm^{-1} originated from stretching vibrations of C-H. The group of C=O was observed at (d) 1,731 cm^{-1} with

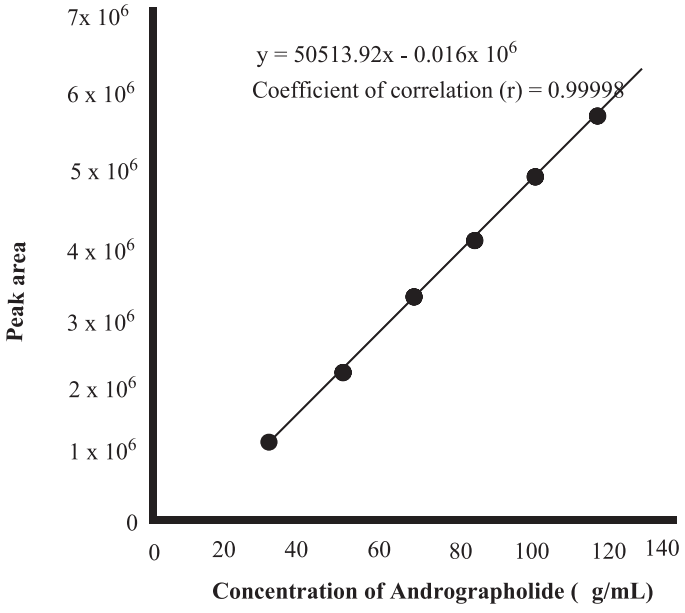

Figure 2. Linear regression curve for correlation between the concentration of andrographolide and area under the curve, as analyzed using HPLC.

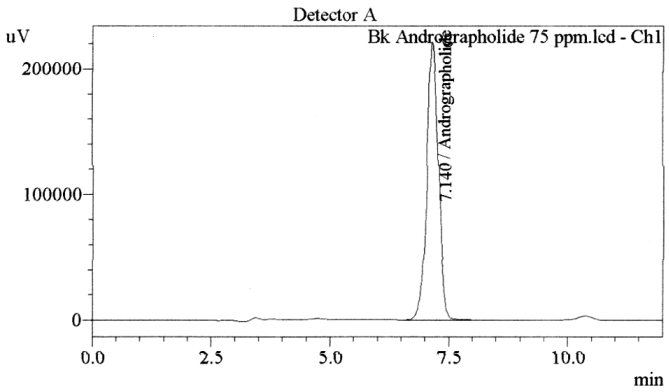

Figure 3. HPLC chromatogram of andrographolide at a concentration of 75 µg/ml. HPLC condition, column: Cosmosil C_{18} column (250 mm × 4.6 mm, 5 µm); mobile phase: methanol : water (60 : 40); flow rate: 0.8 ml/minutes; injection volume: 20 µl; detector: ultraviolet 229 nm.

Table 1. Levels of andrographolide in herb of *A. paniculata* from several regions.

Sample	Concentrations of andrographolide (% *wt/wt*)
AP1	0.5589
AP2	0.4320
AP3	0.9565
AP4	0.5507
AP5	0.7523
AP6	0.4372
AP7	0.5439
AP8	0.7563
AP9	0.5373
AP10	0.9433
AP11	0.6629
AP12	0.3900
AP13	0.6393
AP14	0.5001
AP15	0.3694

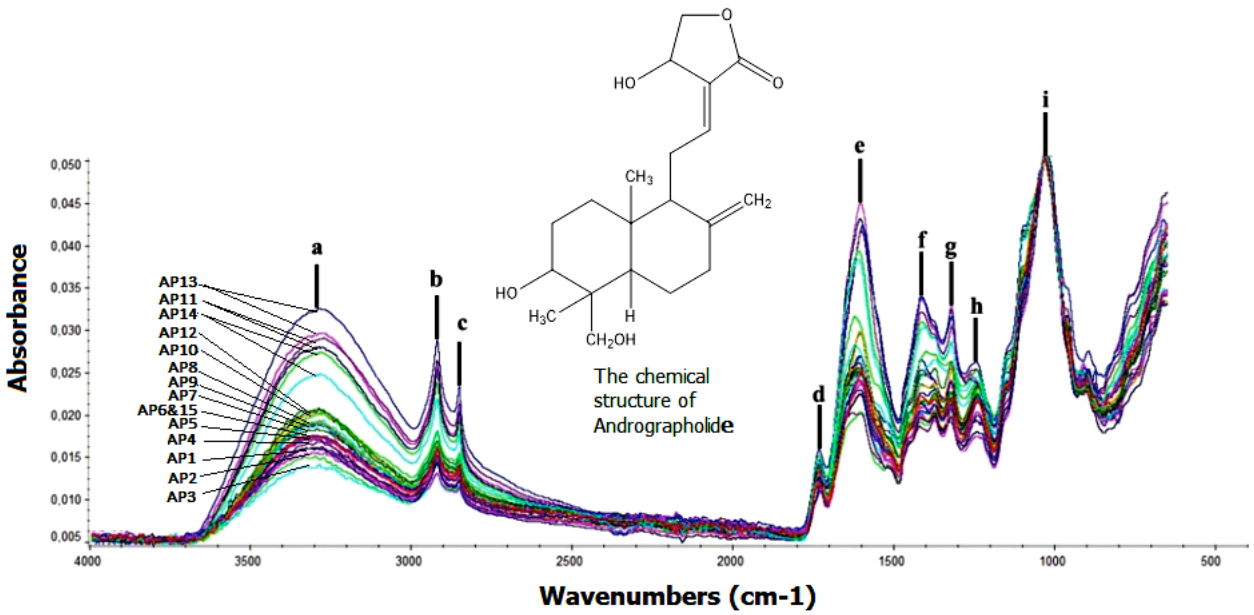

Figure 4. FTIR spectra of dried powder of *A. paniculata* herb from different regions scanned at midwavenumbers of 4,000–650 cm^{-1}. Inset: the chemical structure of andrographolide. AP1–AP3 = *A. paniculata* herb from Bantul, AP4–AP6 and AP15 = *A. paniculata* herb from Sleman, AP7–AP9 = *A. paniculata* herb from Kulon Progo, AP10 and AP12 = *A. paniculata* herb from Semarang, AP11 and AP13 = *A. paniculata* herb from Boyolali, and AP14 = *A. paniculata* herb from Bogor.

stretching vibration mode, while the peaks at (e) 1,605 and (f) 1,416 cm^{-1} were coming from C=C alkenes and bending vibration of CH_2, respectively. The peaks at (g) 1,320 and (h) 1,239 cm^{-1} were originating from C–O in stretching vibration mode. The peak at (i) 1,030 cm^{-1} may be due to the presence of amine C–N stretching vibration (Lestari *et al*., 2017).

Quantitative analysis of ANDR in herbs of *A. paniculata* can be difficult in FTIR spectroscopy due to the overlapping spectra of the molecules in the sample. FTIR spectra combined with multivariate calibrations of PCR and PLSR are useful for the quantitative analysis of analytes in complex mixtures (Rohman, 2014). In PLSR and PCR, the variables used during modeling was absorbance values at specific wavenumbers. The absorbance values were then combined to obtain principal components (PCs) and regressed toward actual values obtained by HPLC analysis

The wavenumbers used were selected based on some variations that existed, especially in peak intensities. The FTIR spectra in normal and derivatization modes were compared for modeling. The derivatization of FTIR spectra could make the overlapping peaks be more resolved, but the sensitivity was decreased (Irnawati *et al*., 2020a). To obtain the best prediction models, the optimizations in terms of the selection of wavenumber regions and the modes of FTIR spectra either in normal or in the first and the second derivatives were optimized (Rohman *et al*., 2015). The selection of optimization parameters was relied on its capability of giving high R^2 and low values of errors, either in calibration models (RMSEC) or in prediction models called RMSEP. The lower errors indicated a more precise model, while the higher R^2 value exhibited the more accurate developed models (Siregar *et al*., 2018).

Table 2 showed the optimization results of FTIR spectra combined with PLSR and PCR for quantitative analysis of ANDR using normal and derivative spectra at specific wavenumbers.

Table 2. The performance of principal PCR and PLSR for quantitative analysis of *A. paniculata* herb.

Multivariate calibrations	Wave number (cm^{-1})	Spectra	Calibration		Validation	
			R^2	RMSEC	R^2	RMSEP
PLS	3,700—665	Normal	0.9894	0.026	0.7444	0.163
		Derivative 1	0.9680	0.046	0.9324	0.068
		Derivative 2	0.9997	0.005	0.9765	0.055
	3,700—2800	Normal	0.9957	0.017	0.4954	0.240
		Derivative 1	0.9999	0.003	0.9361	0.075
		Derivative 2	0.9996	0.005	0.8739	0.085
	1,800—665	Normal	0.9772	0.039	0.6810	0.174
		Derivative 1	0.9480	0.058	0.9075	0.076
		Derivative 2	0.9752	0.040	0.9475	0.077
	3,700—2,800 and 1,800—665	Normal	0.8573	0.093	0.7196	0.115
		Derivative 1	0.9560	0.053	0.9200	0.071
		Derivative 2	0.9990	0.008	0.9667	0.054
PCR	3,700—665	Normal	0.9363	0.064	0.7820	0.106
		Derivative 1	0.9374	0.063	0.8545	0.089
		Derivative 2	0.9519	0.056	0.9127	0.085
	3,700—2800	Normal	0.7368	0.123	0.4065	0.245
		Derivative 1	0.7168	0.126	0.1349	0.167
		Derivative 2	0.7440	0.121	0.2566	0.159
	1,800—665	Normal	0.8783	0.087	0.6742	0.129
		Derivative 1	0.9137	0.074	0.8008	0.101
		Derivative 2	0.9301	0.067	0.8719	0.095
	3,700—2,800 and 1,800—665	Normal	0.7728	0.115	0.3771	0.162
		Derivative 1	0.9054	0.077	0.8165	0.099
		Derivative 2	0.8908	0.092	0.7573	0.109

PLSR using normal spectra at wavenumber region of 3,700–665 cm−1 was used.

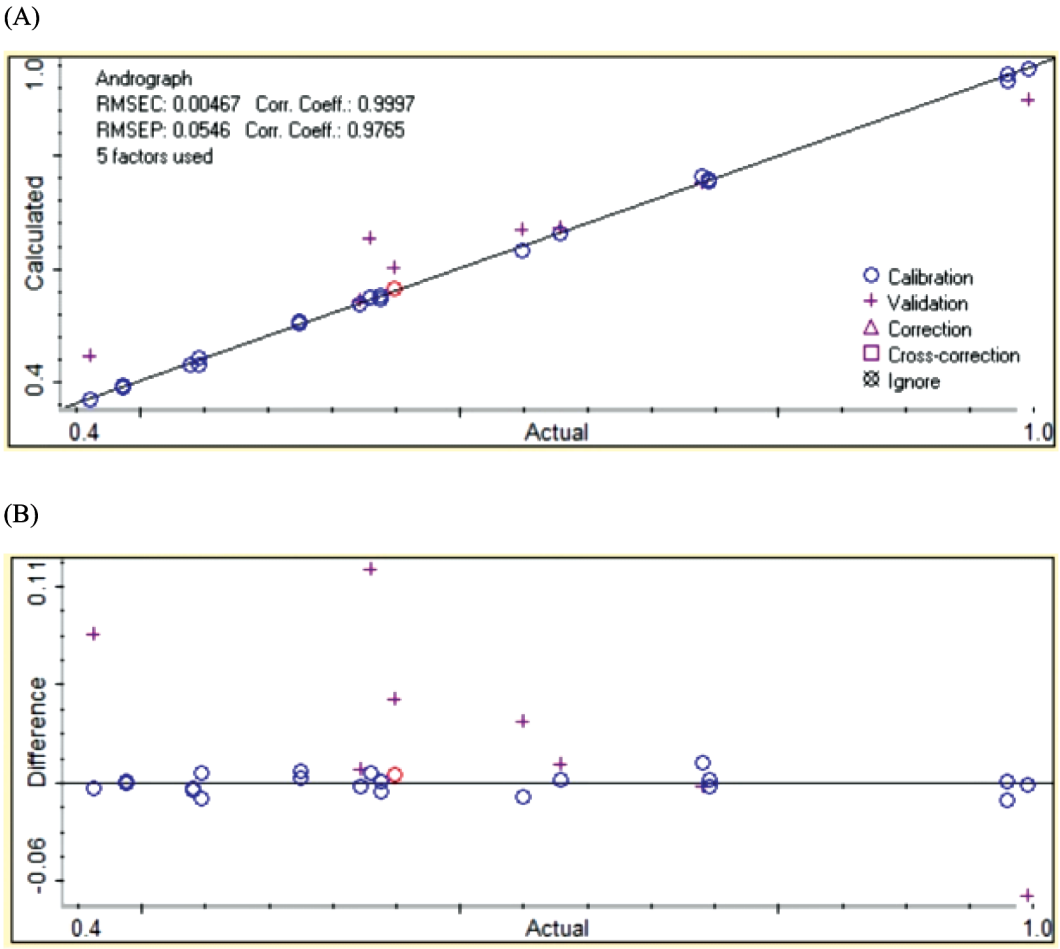

Figure 5. The relationship between actual values (*x*-axis) of andrographolide and the predicted values of andrographolide (*y*-axis) in powdered samples of *A. paniculata* using FTIR spectroscopy (A) along with residual analysis (B.).

These results were expressed by R^2 and RMSEC and RMSEP values. After optimization, PLSR using variables of absorbance values at 3,700–665 cm^{-1} was finally chosen for the prediction of ANDR because this condition could give the highest R^2 values of 0.9997 in the calibration model and 0.9765 in the validation model. The values of RMSEC and RMSEP were relatively low, that isi.e., 0.005% and 0.055%, respectively. These results exhibited that PLSR models offered good accuracy and precision (Miller and Miller, 2010).

Figure 5(A) revealed the scatter plot which explains the correlation between actual (*x*-axis) and predicted values (*y*-axis) of ANDR in powder herbs as determined by HPLC and FTIR spectra with the aid of PLSR using the second derivative FTIR spectra at 3,700–665 cm^{-1}. Figure 5(B) showed a residual analysis of the model, and it indicates the difference between the actual values and predicted values to see the error patterns; therefore, the error that occurred during modeling is negligible because all point differences between actual and predicted value falls above and below zero value. The model developed is reliable to predict ANDR content.

CONCLUSION

The combination of FTIR spectra and PLSR was successfully used for quantification of ANDR using derivative-2 FTIR spectra at 3,700–665 cm^{-1}, with R^2 for the correlation of actual values and FTIR predicted values of 0.9997 (calibration) and 0.9765 (validation), respectively, with RMSEC (0.005%) and RMSEP (0.055%). As a reference, the HPLC method is useful to determine the contents of ANDR in *A. paniculata* herb. The levels of ANDR quantified with HPLC were used as actual values during prediction with FTIR spectroscopy.

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AUTHORS' CONTRIBUTIONS

Hanifah Luthfianasari and Irnawati carried out the research activities, data acquisition, and analyzed data. Abdul Rohman, Sugeng Riyanto, Mohamad Rafi, Bambang Prajogo, Muhammad Bachri Amran designed the research, drafted the manuscript, and made critical thinking on the manuscript.

CONFLICT OF INTEREST

Authors declare that there are no conflicts of interest.

FUNDING

None.

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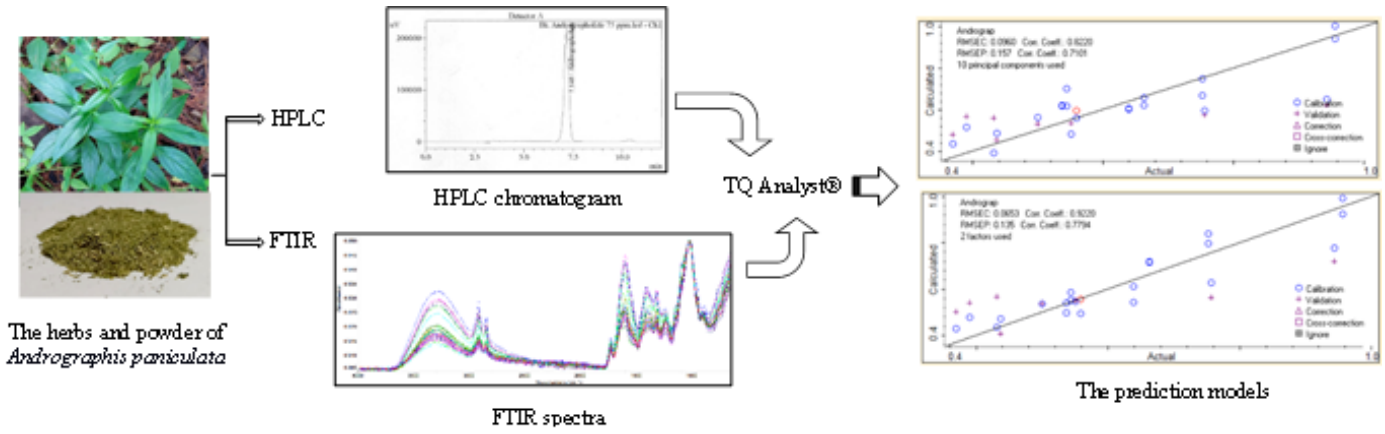
GRAPHICAL ABSTRACT



SUMMARY

Andrographis paniculata or *Sambiloto* in Indonesia has been known to contain ANDR having some biological activities either *in vitro* or *in vivo*. FTIR spectroscopy could be developed as an alternative technique to predict the levels of ANDR without sample preparation during analysis. PLSR is successfully applied for correlating the actual contents of ANDR as quantified using the reference method of HPLC and FTIR predicted values.