Short Communication:

The Use of Chemometrics for Classification of Sidaguri (*Sida rhombifolia*) Based on FTIR Spectra and Antiradical Activities

Abdul Rohman[1,2*], Asefin Nurul Ikhtiarini[2], Widiastuti Setyaningsih[3], Mohamad Rafi[4], Nanik Siti Aminah[5], Muhamad Insanu[6], Irnawati Irnawati[7], and Djoko Santosa[8]

[1]*Center of Excellence Institute of Halal Industry and Systems, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

[2]*Department of Pharmaceutical Chemistry Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta 55281, Indonesia*

[3]*Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

[4]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, IPB University, Jl. Tanjung Kampus IPB Dramaga, Bogor 16680, Indonesia*

[5]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Airlangga, Jl. Mulyorejo Kampus C Unair, Surabaya 60286, Indonesia*

[6]*Pharmaceutical Biology Research Group, School of Pharmacy, Institute Technology Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia*

[7]*Faculty of Pharmacy, Halu Oleo University, Kendari Indonesia*

[8]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia*

* ***Corresponding author:***

email: abdul_kimfar@ugm.ac.id

Received: February 26, 2021
Accepted: May 3, 2021

DOI: 10.22146/ijc.64360

Abstract: *Sidaguri (*Sida rhombifolia*) is one of the herbal components used in traditional medicine. The application of chemometrics in the standardization of herbal medicine is common. The objective of this study was to classify Sidaguri from different regions based on FTIR spectra with chemometrics of principal component analysis (PCA) and to correlate the antioxidant activities with FTIR spectra using the multivariate calibration of partial least square regression (PLSR). The extraction of Sidaguri powder was performed using ultrasound-assisted extraction (UAE) at optimum conditions. The obtained extracts were subjected to antiradical scavenging activities using DPPH (2,2'-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) radicals. The PCA result shows that Sidaguri from different regions could be separated using 14 wavenumbers of FTIR spectra based on the PCA's loading plot. PLSR regression using the second derivative FTIR spectra at wavenumbers of 3662–659 cm^{-1} could predict radical scavenging activities (RSA) of Sidaguri with R^2 values of 0.9636 and 0.9024 for calibration and validation models, with RMSEC and RMSEP values of 1.45% and 2.65%, respectively. It can be concluded that FTIR spectra treated by PCA were reliable for classifying Sidaguri from different regions. At the same time, PLSR was accurate and precise enough to predict the RSA of Sidaguri.*

Keywords: *Sidaguri; herbal standardization; principal component analysis; radical scavenging activities; partial least square regression*

■ INTRODUCTION

Sidaguri (*Sida rhombifolia*) is one of the herbal components used in Indonesian traditional medicine [1]. Many studies reported that Sidaguri extracts provided potent free radical scavenging activity towards 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical, 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radical, reducing power, superoxide scavenging activity, antibacterial activity, and anti-inflammatory activities [2]. These biological activities are manifestations of secondary metabolites, bioactive compounds such as flavonoids, and tannins contained in Sidaguri [3]. Furthermore, the production of the bioactive compounds was significantly affected by extrinsic factors such as climate, temperature, sun exposure, rainfall, and soil type [4]. Thus, Sidaguri contained various metabolites which are beneficial to human health. Therefore, it is necessary to analyze those metabolites and bioactive compounds expressed by antioxidant activities for controlling its quality comprehensively.

Fourier transforms infrared (FTIR) spectroscopy is a vibrational spectroscopies widely applied to standardize herbal medicines due to its property as a fingerprint analytical technique [5]. Combined with multivariate data analysis (chemometrics), FTIR spectra have been used for the classification of herbal components such as *Dendrubium officinale* [6], *Radix puerariae* [7] and *Curcuma* species [8-10]. In the last decades, chemometrics for the standardization and quality control of herbal medicines is frequently reported by classifying the herbal medication or predicting certain compounds [11-13]. However, using a literature study, there is no report regarding applying the combination of FTIR spectra and chemometrics for the classification of Sidaguri.

The metabolites of Sidaguri vary depending on their geographical origin. Thus the quality control of Sidaguri is needed. One of the analytical techniques widely used for metabolite fingerprinting analysis is FTIR spectroscopy, especially in combination with chemometrics. FTIR spectroscopy possesses several advantages compared to other analytical methods. It is a non-destructive technique capable of perceiving the functional groups and provides information about structural and chemical changes [14]. The objectives of this study were to classify Sidaguri based on FTIR spectra with chemometrics of principal component analysis (PCA) and correlate the antioxidant activities with FTIR spectra using chemometrics of partial least square regression (PLSR).

■ EXPERIMENTAL SECTION

Materials

The samples of Sidaguri were taken from several regions in the Province of Central Java (Manisrenggo, Borobudur, and Mungkid) and Daerah Istimewa Yogyakarta (Depok, Imogiri, Kretek, Cangkringan, and Ngempak), Indonesia. Distilled water and purified water were purchased from PT. Ikapharmindo Putramas, Indonesia. The plant authentication of Sidaguri was performed in the Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, under the supervision of botanist Dr. Djoko Santosa. Methanol for analysis was purchased from E. Merck (Darmstadt, Germany), 2,2'-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), potassium persulfate, and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma (Aldrich, USA).

Procedure

Preparation of extract

The leave powder weighed as much as 0.5 g and placed in a 30 mL vial, and is extracted using ultrasonic-assisted extraction (UAE) using probe Hielscher UP200ST according to [15] with slight modification. This ultrasonic probe had an ultrasonic frequency of 24 kHz. The extraction condition was obtained by evaluating four factors: solvent to solid ratio, extraction temperature, MeOH concentration (in distilled water), and sonication. The sonication process was done using Box-Behnken Design (BBD) with variable response 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. The optimum condition of UAE used the ratio of solvent to powdered samples (26:1 v/weight), extraction temperature of 45.45 °C, methanol concentration of 42%, power of sonication of 86%, and

time extraction of 5 min. In addition, the obtained extract was subjected to antioxidant activity.

FTIR spectra measurement

The scanning of FTIR spectra of Sidaguri powder was carried out using an FTIR spectrophotometer (Thermo Scientific Nicolet iS10, Madison, WI), controlled with the software Omnic, according to Martono et al. [16]. The measurements were carried in the mid-infrared region of 4000–650 cm^{-1} with 32 scannings with a resolution of 8 cm^{-1} using horizontal attenuated total reflectance (HATR) composed of ZnSe crystal. All FTIR spectra were corrected against the FTIR spectrum of air as background. After every scan, a new reference air background spectrum was taken. In addition, FTIR spectra were recorded as absorbance values at each data point in triplicate to facilitate quantitative research.

DPPH radical scavenging assay

DPPH radical scavenging assay was carried out according to Antasionas et al. [17] with slight modification. First, a-0.5 g of extract was dissolved in the methanol-water mixture (42:58 v/v) until 100.0 mL. Then, a-100 µL solution was added to 2.9 mL DPPH (91.296 µmol dissolved in methanol). The solution is incubated for 50 min in a dark place at room temperature. The absorbance of the solution is measured using a spectrophotometer (Genesys 10S UV-Vis, Japan) at a maximum wavelength of 516 nm. The percentage of radical scavenging activity (% RSA) is calculated as:

$$\%RSA = \frac{\text{abs. of blank} - \text{abs. of sample}}{\text{abs. of blank}} \times 100\% \quad (1)$$

The blank used was 2.9 mL DPPH 100 µL of methanol/water mixture and treated as the sampling procedure and recorded at 516 nm.

ABTS radical cation decolorization assay

$ABTS^{\cdot+}$ assay was performed according to Antasionas et al. [17] with slight modification. This assay was carried out by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution allows the mixture to stand in the dark at room temperature for 12 to 16 h before use. The procedure is performed by adding 30 µL of extract solution into 3 mL of the $ABTS^{\cdot+}$ radical cation solution (diluted with methanol at a ratio of 1:10). The absorbance was recorded at 731 nm against blank after 6 min. The standard curve is prepared using 7 different concentrations of Trolox (2, 5, 7, 10, 12, 15, and 17 mg/L) and treated as the same sample procedure. The results were expressed as mg Trolox Equivalent per gram Sidaguri powder (mg TE/g sample).

Data analysis

Absorbance value in selected wavenumbers was used as variables to classify Sidaguri powder by principal component analysis (PCA). In addition, absorbance values were used as variables correlated with antioxidant activity by partial least square (PLS). Data analyses by PCA and PLS were carried out using Minitab version 17 (USA) and TQ analyst software (Thermo Scientific Nicolet iS10, USA), respectively. In PCA, the classification of samples can be obtained by evaluating the score plot, while PLS was used to predict the radical scavenging activities using the predictor variable of FTIR spectra.

■ RESULTS AND DISCUSSION

Metabolite fingerprinting based on FTIR spectra were applied to analyze Sidaguri powder obtained from 8 regions. Sidaguri powder was used instead of Sidaguri extract to avoid bias in FTIR analysis due to water residue in the extract. The presence of water will result in the stretching vibration of –OH at a wavenumber of 3200–3500 cm^{-1}. Fig. 1 exhibited FTIR spectra of Sidaguri powder scanned at the whole mid-infrared region of 4000–650 cm^{-1}. Each peak and shoulder in FTIR spectra corresponded to functional groups responsible for infrared absorption. The functional groups present in the evaluated samples were compiled in Table 1. The pattern of FTIR spectra of the evaluated Sidaguri powders is quite similar to each other. Therefore, it provided information on similar metabolites contained in the samples being assessed. Nonetheless, the signal intensities of those spectra patterns look a bit different due to the different levels of metabolites contained in the samples. In addition, some extrinsic factors such as soil type, climate, humidity, rainfall, and sun exposure contribute to the types and concentration of secondary metabolites [18].

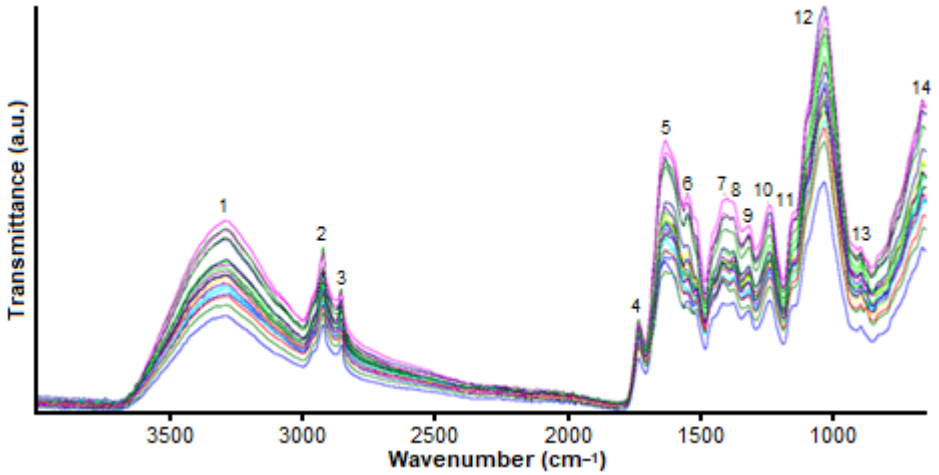

Fig 1. FTIR spectra of Sidaguri powder scanned using attenuated total reflectance at wavenumbers of 4000–650 cm^{-1}

Table 1. Functional groups were responsible for absorbing IR spectra in each peak and shoulders in Sidaguri [24-25]

Annotation	Wavenumber (cm^{-1})	Functional group, modes of vibration
1	3283	O–H carboxylic acids stretch, N–H 1' and 2' amine stretch, amide stretch
2	2920	C–H alkane stretch, C–H aldehyde stretch
3	2850	C–H alkane stretch, C–H aldehyde stretch
4	1732	C=O carbonyls, acid, anhydride, acyl chloride, ester, amide, aldehyde, and ketone stretch
5	1634	C=C alkene stretch, N–H 1' amine bend
6	1550	C=C alkene stretch, N=O nitro compound asymmetric stretch, N–H 1' amine bend,
7	1415	C–H alkane bend
8	1372	N=O nitro compound symmetric stretch, C–H alkane bend
9	1315	N=O nitro compound symmetric stretch
10	1238	C–O esters, ethers, alcohols, carboxylic acids stretch, C–N aliphatic amines stretch, C–H haloalkane wog
11	1153	C–O esters, ethers, alcohols, carboxylic acids stretch, C–N aliphatic amines stretch
12	1130	C–O esters, ethers, alcohols, carboxylic acids stretch, C–N aliphatic amines stretch
13	898	N–H 1' and 2' amine wog, C–H alkene bend
14	663	N–H 1' and 2' amine wog, C–H alkene bend, C–X alkyl bromide stretch

The classification of Sidaguri could not be accomplished by only observing their raw spectra. Therefore the chemometrics of principal component analysis (PCA) was used. PCA is one of unsupervised pattern recognition widely used for the classification of objects. PCA could reduce the dimensionality of original data by retaining the variance in the original data. The output of PCA, namely principal components (PCs), is also called latent variables in which samples with the same first principal component (PC1) and the second principal components (PC2) values are considered as the same [19]. PCA provides information towards similarities and differences of all of the samples displayed by the score plot. In this study, the absorbance values at 14 different peaks of FTIR spectra were used as variables for profiling 8 other Sidaguri powders. The use of these absorbance values during PCA was based on a high loading plot. The higher loading of predictor variables indicated that those are more contributing than the lower one. The score plot of PCA in Fig. 2 accounts for 97.2% of the total variance, with PC1 and PC2 explained 92.6% and 4.7% of the variances, respectively.

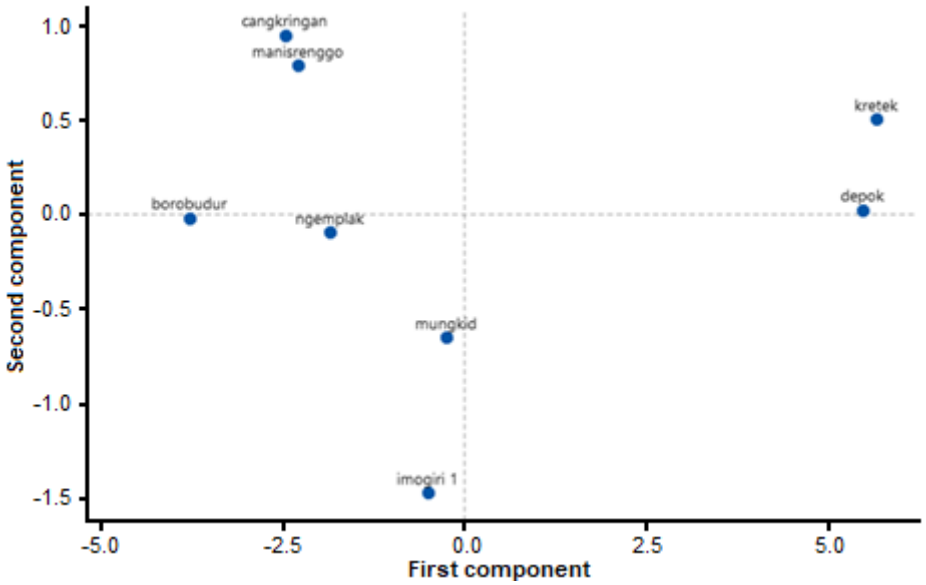

Fig 2. PCA score plot of Sidaguri from 8 regions (Depok, Imogiri, Kretek, Manisrenggo, Cangkringan, Ngemplak, Bodobudur, Mungkid)

PCA loading plot provided information about the variables that are most contributing to the sample separation. The more a variable is away from zero axis point (0.0), the higher it is donated to the separation [20]. Thus, absorbance values at 3283 and 1732 cm^{-1} were the most significant variables in PC 1 and PC 2, respectively (Fig. 3). The loading plot could be applied to evaluate the correlation among variables. The coincided angle created by variables indicated a high correlation among variables. Therefore, it is clear that all variables are highly correlated to each other (Fig. 3).

Antioxidant activities of Sidaguri as determined using DPPH radical assay and TEAC assay were compiled in Table 2. Partial least square regression (PLSR) was carried out to create the regression which correlates the absorbance values (predictor variables) and the antioxidant activities (response variables). PLSR model could be achieved using normal FTIR spectra or

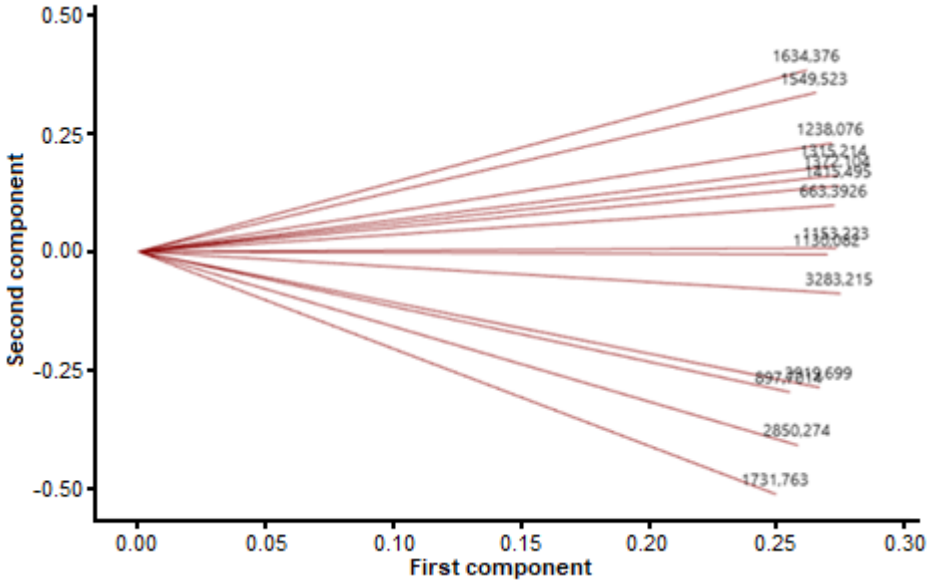

Fig 3. PCA loading plot of PC1 and PC2 for classification of Sidaguri from 8 regions

Table 2. The antioxidant activities of Sidaguri powder as determined using DPPH radical assay and TEAC assay

Regions	DPPH assay (RSA %)	TEAC assay (mg TE/100 g Sidaguri powder)
Depok	79.73 ± 0.60	231.15 ± 33.28
Imogiri	65.51 ± 1.38	194.60 ± 6.51
Kretek	83.69 ± 0.30	216.16 ± 10.57
Manisrenggo	81.03 ± 0.23	255.76 ± 13
Cangkringan	81.94 ± 0.44	285.04 ± 59.34
Ngemplak	82.95 ± 0.47	320.81 ± 15.09
Borobudur	79.35 ± 0.99	227.91 ± 7.78
Mungkid	81.01 ± 0.36	245.0 ± 5.13

derivative FTIR spectra. The first derivative FTIR spectra eliminate the general intensity effect and simplify the baseline selection, while the second derivative FTIR spectra remove the slope effect. Derivative spectra also enhance the separation of overlapping absorption bands. However, derivative spectra also provide a lower detection limit than standard spectra [21]. Table 3 showed the PLS model along with statistical parameters, namely coefficient of determination (R^2) for calibration and validation, root mean square error calibration (RMSEC), and root mean square error prediction (RMSEP) values. Those parameters were responsible for correlating the actual antioxidant activity values by DPPH assay. They predicted FTIR spectra to obtain the unknown antioxidant activity value using normal, first derivative, and second derivative spectra at the specific wavenumbers (1781–1187, 1301–911, 3001–2779, 3001–1478, and 3662–359 cm^{-1}). The model was chosen to predict the antioxidant activity based on the capability of the PLS model for providing the highest R^2 value and lowest RMSEC and RMSEP. RMSEC is a parameter used to evaluate the errors in the calibration model. Meanwhile, RMSEP is used to assess the validation model. In modeling multivariate calibration, the validation procedure is necessary to check overfitting (the model had a high correlation with a small error value in calibration datasets, but it could not provide a good result for another dataset) [22].

Table 3. Multivariate calibration of partial least square for prediction radical scavenging activity in Sidaguri

Wavenumber (cm^{-1})	Spectra	Calibration		Prediction	
		RMSEC (%)	R^2	RMSEP (%)	R^2
1781-1187	Normal	5.33	0.1886	5.08	0.4655
	1st derivative	4.66	0.5143	4.44	0.5995
	2nd derivative	4.58	0.5374	4.25	0.6458
1301-911	Normal	5.40	0.1109	5.24	0.3845
	1st derivative	4.47	0.5685	4.91	0.5260
	2nd derivative	0.498	0.9958	3.79	0.8667
3001-2779	Normal	3.64	0.7420	2.82	0.8703
	1st derivative	4.89	0.3965	4.84	0.5628
	2nd derivative	2.59	0.8790	4.50	0.5865
3001-1478	Normal	5.20	0.2864	4.83	0.5434
	1st derivative	3.11	0.8194	3.71	0.7665
	2nd derivative	1.86	0.9392	2.82	0.8835
3662-359	Normal	5.33	0.1850	5.09	0.4562
	1st derivative	2.91	0.8446	3.87	0.7503
	2nd derivative	**1.45**	**0.9636**	**2.65**	**0.9024**

*the selected condition was marked with **bold**

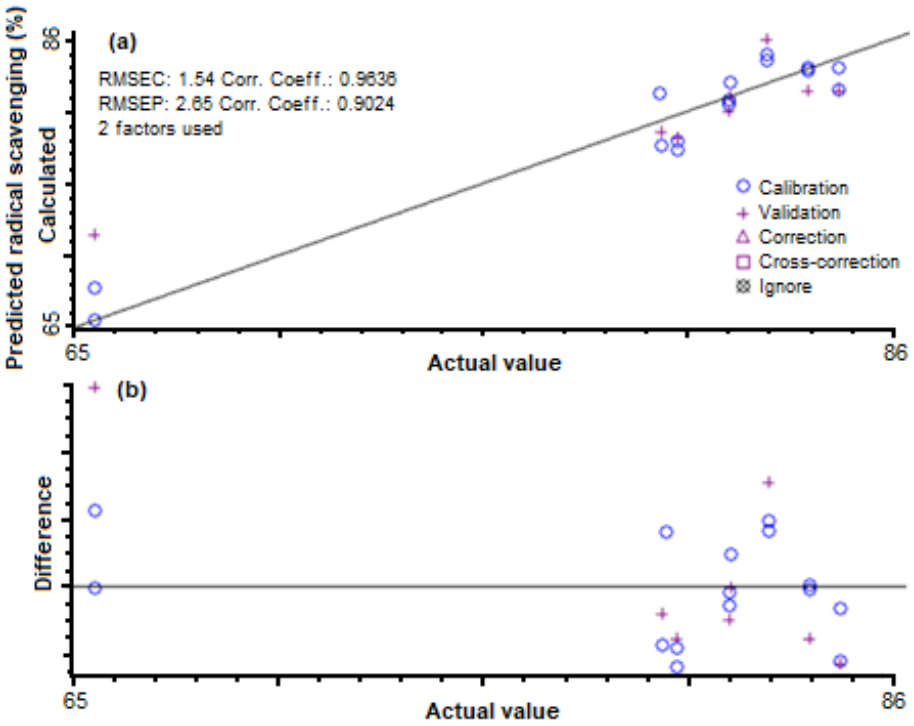

Fig 4. PLS calibration model for predicting the antioxidant activities in Sidaguri. [a] = calibration model for the correlation between actual and predicted values; [b] = the difference between actual and predicted values (residual analysis)

Based on statistical parameters in Table 3, the second derivative FTIR spectra at the wavenumber region of 3662–659 cm^{-1} were selected to predict the antioxidant activities in Sidaguri with an R^2 value of 0.9636 and 0.9024 in calibration and validation models. In contrast, RMSEC and RMSEP values obtained were 1.45% and 2.65%, respectively. The lower value of RMSEC and RMSEP, the more precise the PLS model. Fig. 4 exhibited the correlation plot between actual and FTIR predicted values of DPPH radical assay (a) along with residual analysis (b). From residual analysis, the difference between the actual and predicted value is around zero. Therefore it can be concluded that modeling systematic error did not come from the PLS modeling between the actual value of antioxidant activities and FTIR predicted value [23]. Therefore, from R^2 values, RMSEC, and RMSEP values, along with residual analysis, it can be stated that FTIR spectroscopy combined with PLSR is accurate and precise enough for predicting antiradical scavenging activities of Sidaguri.

■ CONCLUSION

Attenuated total reflectance-FTIR spectroscopy (ATR-FTIR) employing absorbance values as variables combined with PCA based on PC1 and PC2 score plots could classify Sidaguri from different regions with clear separation. Furthermore, PLSR using the second derivative FTIR spectra at wavenumbers of 3662–659 cm^{-1} could predict radical scavenging activities with acceptable accuracy and precision. Thus, the developed method is rapid and reliable and can be further used to standardize herbal medicine.

■ ACKNOWLEDGMENTS

This study was supported by *Riset Kolaborasi Indonesia* (RKI) 2021, Directorate of Research, Universitas Gadjah Mada, Indonesia.

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