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Prediction of antioxidant activities of Sidaguri (*Sida rhombifolia*) with different harvesting times using FTIR spectra and chemometrics

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Abstract

Sidaguri (*Sida rhombifolia*) is one of the compound plants widely used as herbal components and is reported to contain some bioactive responsible for biological activities including antioxidants. This study aimed to evaluate the antioxidant activities of Sidaguri from different harvesting times and to correlate FTIR spectroscopy in combination with chemometrics for the prediction of antioxidant activities. Sidaguri was planted and cultivated for the third, fourth, and fifth months and then evaluated for its antioxidant activities, phenolic contents, and flavonoid contents. Sidaguri samples were also subjected to FTIR spectral measurement to be correlated with scavenging activity toward 2,2′-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid. The results showed that the longer harvesting time tends to lower the antioxidant activities which can be associated with the lower bioactive components responsible for antioxidant activities. Partial least square applied to 1st derivative FTIR spectra using absorbances at wavenumbers of 3700-650 cm^{-1} provide the best modelling for correlation between actual values of antioxidant and predicted values with R^2-calibration and R^2-validation of 0.9995 and 0.9998, with the root mean square error of calibration (RMSEC), the root mean square error of calibration (RMSEP) values of 0.0674 and 0.0827, PRESS and the root-mean-square error of cross-validation (RMSECV) values 0.226 and 0.194, respectively. The chemometrics of principal component analysis (PCA) could separate Sidaguri samples harvested during different months. FTIR spectra in combination with chemometrics could be an alternative method for the prediction of antioxidant activities of Sidaguri with acceptable accuracy and precision.

1. Introduction

In recent years, the use of herbs as a component of traditional medicine has increased tremendously due to some reasons including safety, availability, and effectiveness. Herbal-based medicines have been associated with preventing some degenerative diseases such as cancer, diabetes mellitus, and oxidative stress-related diseases (Rohman *et al*., 2020). Sidaguri with the scientific name of *Sida rhombifolia* is one of the herbal medicines, belonging to the family of Malvaceae which has been widely applied for the treatment of certain diseases for thousands of years including headaches, tuberculosis, diabetes, malaria, hemorrhoids, wounds, rheumatic, cardiac disease, diarrhoea, and skin diseases (Ikhtiarini *et al*., 2021). Sidaguri contains some bioactive compounds responsible for these biological activities including phenolic compounds such as phenolic acids and flavonoids, coumarin, and alkaloids (Chaves *et al*., 2017; Ferro *et al*., 2019). The phenolic contents are known as potent antioxidant activities, therefore, the

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evaluation of antioxidant activities of Sidaguri along with its correlation with phenolics and flavonoids are needed.

Food and pharmaceutical industries typically use synthetic antioxidants with exact chemical composition; however, some regulations restricted their use due to the same reasons, especially safety issues, therefore, natural antioxidants derived from plants are extensively explored recently to obtain the plant antioxidant comparable to synthetic antioxidants (Dhalwal *et al*., 2007; Embuscado, 2015). Antioxidants are typically defined as any substances significantly capable of delaying or preventing oxidation reactions. The antioxidant activities are typically assessed using some mechanisms such as radical scavenging using 2,2'-diphenyl-1-picrylhydrazyl, Trolox equivalent antioxidant capacity (TEAC) method, Ferric reducing activity power (FRAP), lipid peroxidation inhibition assay using beta-carotene bleaching method and linoleate-thiocyanate method, and metal chelating activity (Herlina *et al*., 2018; Widodo *et al*., 2020). DPPH radical scavenging assay and TEAC are the most popular methods using the mechanism of radical scavenging among others, however, this method involves the use of reagents and solvents which are pollutants to the environment, as a consequence, the green analytical method based on spectroscopic measurement could be proposed as an alternative method for analysis of antioxidant activities.

FTIR spectroscopy is a powerful analytical method capable of obtaining big data of absorbance values, even from a single measurement. This method can be used for the direct measurement of samples without any sample preparation step. Using attenuated total reflectance (ATR) mode, the herbal samples (powder, extracts) could be directed scanned to obtain FTIR spectra (Singh *et al*., 2010). The combination of FTIR spectra and chemometrics offered a fast and reliable method for the prediction of antioxidant activities and their correlation with chemometrics (Tejamukti *et al*., 2020). This combination has been successfully applied for the prediction of antioxidant activities of *Baccaurea racemosa* and *Macaranga subpeltata* leaves (Widodo *et al*., 2020). However, using a literature study, there is no reported publication on the application of FTIR spectra for the prediction of antioxidant activities of Sidaguri. Therefore, the objectives of this study were to evaluate the antioxidant activities of Sidaguri from different harvesting times and to correlate FTIR spectroscopy in combination with chemometrics of multivariate calibration of partial least square and principal component regression for the prediction of antioxidant activities.

2. Materials and methods

2.1 Sample preparation

The Sidaguri herb powder harvested in the third, fourth, and fifth months each weighed 1 g, was placed in a 100 mL beaker, and added 25 mL of solvent consisting of 42% methanol in distilled water, then the mixture was extracted with an ultrasonic bath frequency of 50 Hz for 5 mins. The mixture was then allowed to stand for 24 hrs accompanied by shaking and stirring occasionally. Then filtered with Whatman filter paper. Samples were subjected to antioxidant activity, total flavonoid, and total phenolic content (Ikhtiarini *et al*., 2021; modified).

2.2 DPPH radical scavenging assay

The antioxidant activity test was carried out using a modified method from Akbar *et al*. (2020). The sample was weighed as much as 1 g, and then the powder was dissolved in 25 mL of methanol 42% to obtain a sample with an initial concentration of 40 mg/mL (stock solution). Then, the stock solution was pipetted as much as 20, 40, 60, 80, 100, 120 and 140 µL into different 5 mL volumetric flask to get samples with concentrations of 0.16, 0.32, 0.48, 0.64, 0.8, 0.96 and 1.12 mg/mL. The solutions were then added with 1 mL DPPH 0.4 mM in a volumetric flask and made into marks to 5 mL with methanol. The mixture was homogenized by vortex and allowed to stand at room temperature and darkroom place for 35 mins. The absorbance was measured using a spectrophotometer with the maximum wavelength (515 nm) against the blank consisting of sample and methanol. Then, 1 mL of DPPH 0.4 mM and 4 mL of methanol were used as a control.

The preparation of the Trolox stock solution was weighed as much as 12.6 mg and then dissolved in methanol to 25 mL. The concentration series solution was prepared by pipetting 20, 40, 60, 80, 100, 120, and 140 µL, then added 1 mL of 0.4 mm DPPH and added methanol to 5 mL in a measuring flask to get concentrations of 2, 4, 6, 8, 10, 12 and 14 µg/mL. All concentration series solutions were homogenized using a vortex and allowed to stand at room temperature and in a dark place for 35 mins, then read the absorbance at a wavelength of 515 nm using a spectrophotometer. The percentage of the antioxidant activity or percent inhibition is calculated using the equation:

$$\%Antioxidant\ Activity = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

The IC_{50} value is calculated using a linear equation obtained from the correlation between the concentration and percentage of antioxidant activity.

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2.3 Trolox equivalent antioxidant capacity

$ABTS^{\bullet+}$ assay was performed according to Rajurkar and Hande (2011) with slight modification. Solution of ABTS (7 mM) and potassium persulfate (2.4 mM) was mixed to make the stock solution and placed in the dark for 12-16 hours at room temperature. The procedure was carried out by adding 100 µL of extracted Sidaguri and 500 µL of the $ABTS^{\bullet+}$ radical cation solution and adding methanol to 5.0 mL. The absorbance was recorded at 745 nm against a blank (sample and methanol) after 6 mins.

Making a standard Trolox curve by pipetting 20, 40, 60, 80, 90, 100, and 120 µL, 500 µl of $ABTS^{\bullet+}$ cation radical solution was added, and the volume was adjusted to 5 mL with methanol to get with concentrations of 2, 4, 6, 8, 10, 12, and 14 µg/mL. The absorbance was measured with a spectrophotometer (UH-5300 Hitachi, Japan) at a wavelength of 745 nm after all the concentration series solutions had been allowed to stand for 6 mins.). The result was expressed as mg Trolox equivalent antioxidant capacity (TEAC)/g Sidaguri powder.

2.4 Total flavonoid content assay

Determination of total flavonoid content (TFC) was carried out according to Yulia *et al*. (2019) and Christova -Bagdassarian *et al*. (2013) with slight modifications**.** The solution of Sidaguri extract was pipetted as much as 0.3 mL and then dissolved with 3 mL of methanol in the 10 mL volumetric flask, added with a reagent consisting of 0.2 mL of 10% $AlCl_3$, 0.2 mL of CH_3COOK, and the total volume was made up to 10 mL with add distilled water, vortexed homogeneously, subjected to incubation for 30 mins at room temperature. The absorbance was measured against the reagent blank at 433 nm using UV-VIS Spectrophotometer (UH-5300 Hitachi, Japan).

The quercetin standard solution was 0.5 mg/mL, then pipetted as much as 40, 80, 120, 160, 200, 240, and 280 µL, then added 3 mL of methanol, 0.2 mL of 10% $AlCl_3$, 0.2 mL of potassium acetate, and added distilled water to 10 mL in a volumetric flask to get with concentrations of 2, 4, 6, 8, 10, 12, and 14 µg/mL. All solutions were homogenized using a vortex and then allowed to stand for 30 mins. The absorbance was noted at 510 nm using a UV-Visible spectrophotometer (UH-5300, Hitachi, Japan). FC is expressed as mg quercetin equivalent/g Sidaguri powder.

2.5 Total phenolic content assay

Total phenolic content (TPC) in Sidaguri extract was determined according to Folin-Ciocalteau (FC) method, according to the method by Sidhiq *et al*. (2020) and Ikhtiarini *et al*. (2021) with slight modifications. A-0.1 mL of Sidaguri extract was placed in a 10 mL volumetric flask and added with 0.4 mL of n Folin Ciocalteau reagent, vortexed homogeneously, allowed to stand for 5 mins, added with 4 mL of Na_2CO_3 and 5.5 mL of distilled water. The solution was incubated for 35 mins at room temperature and the absorbance was read against the reagent blank at 739 using UV-VIS Spectrophotometer (UH-5300 Hitachi, Japan).

The standard solution of gallic acid 0.5 mg/mL ppm was pipetted as much as 40, 80, 120, 160, 200, 240, and 280 µL, then added 4 mL of Folin Ciocalteau, shaken and allowed to stand for 5 mins, then added 4.0 mL of 7% Na_2CO_3 solution and made up to 10 mL of distilled water in a volumetric flask to get concentrations of 2, 4, 6, 8, 10, 12, and 14 µg/mL. All solutions were homogenized by vortex, allowed to stand for 35 mins, and absorbance was measured using a spectrophotometer at a wavelength of 739 nm. The results were expressed as mg gallic acid equivalent/g of Sidaguri powder.

2.6 FTIR spectra measurement

The scanning of FTIR spectra of Sidaguri powder was carried out using an FTIR spectrophotometer (Thermo Scientific Nicolet iS10, Madison, WI). The scanning was controlled with the software Omnic, according to Martono and Rohman (2019). The measurements were carried out in the mid-infrared region of 4000–650 cm^{-1} with 32 scanning 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. In addition, FTIR spectra were recorded as absorbance values at each data point in triplicate to facilitate a quantitative study based on Lambert-Beer law.

2.7 Data analysis

The antioxidant activities were expressed as mean and standard deviation and calculated with the aid of Excel (Microsoft Inc., USA). The multivariate calibrations of PLS and PCR for making the correlation between actual values of antioxidant and FTIR spectra were managed using TQ Analyst software included in the FTIR spectrophotometer instrument. The statistical parameters used in multivariate calibrations included coefficient of determination (R^2) for accuracy and root mean square error of calibration (RMSEC) and prediction (RMSEP) for precision evaluation. Principal component analysis (PCA) for clustering of Sidaguri samples from different harvesting times was carried out by Minitab software version 17.

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3. Results and discussion

3.1 Antioxidant activities of Sidaguri

Table 1 compiles the antioxidant activities of Sidaguri powder as evaluated by radical scavenging activities using DPPH radical assay and Trolox equivalent antioxidant capacity (TEAC). Sidaguri samples were taken from the plantation at different harvesting times (third, fourth, and fifth) and the results indicated that the longer the cultivation, the lower the radical scavenging. It can be deduced that the longer harvesting time of Sidaguri tends to reduce the levels of bioactive components (phytochemicals) responsible for the antioxidant activities. This can be seen from the reduced levels of total flavonoid content (TFC) and total phenolic contents (TPC) of Sidaguri samples as the function of harvesting times. Similar results were observed in which the antioxidant activities of olive oils decreased with harvesting times (Özcan *et al*., 2019). It is also reported that the antioxidant activities of sorghum were reduced with the longer harvesting time, therefore, the earlier harvesting of sorghum is performed to improve its antioxidant effects (Jeon *et al*., 2017) has been improved.

The reduced antioxidant activities of Sidaguri with harvesting time were also observed for the levels of TPC and TFC, therefore, a loading plot of principal component analysis (PCA) was applied to make such a correlation. Some studies have reported a good correlation between antioxidant activities with TPC and TFC of the extracts from various plants, and in this study, the chemometrics analysis of PCA was used for this purpose (Makarova *et al*., 2021). In PCA's loading plot, the lines indicated how the vectors are pinned from the origin of PC1 and PC2 = 0 expressing the weight of each variable to PCs. If two vectors form small angles, the two variables are positively correlated. If variables form an angle of about 90°, they are not likely to be correlated. If two variables are diverged and form a large angle (about 180°), a negative correlation occurs (Widodo *et al*., 2019). Figure 1 reveals the loading plot of PCA using variables of IC_{50} values of DPPH radical scavenging activity, TEAC, TPC and TFC. There is a positive relationship between TEAC and TPC with a Pearson correlation value of 0.912. The results of antioxidant activities using DPPH radical scavenging methods were linear in which the low IC_{50} values of samples had the higher TEAC.

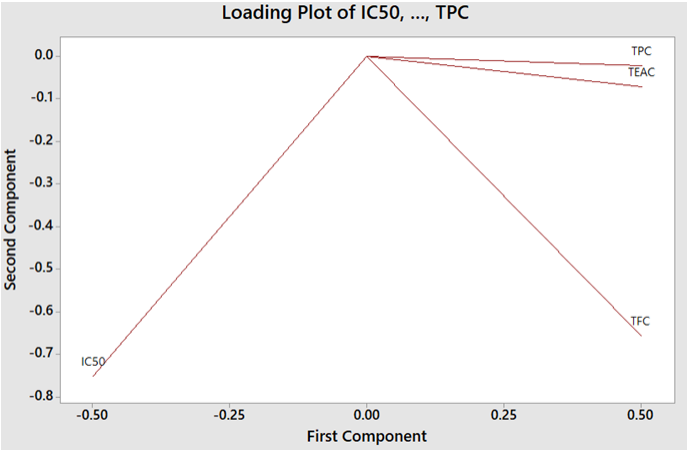

Figure 1. The loading plot of PCA using variables of IC_{50} values of DPPH radical scavenging activity, TEAC, TPC and TFC.

In order to classify Sidaguri samples based on different harvesting times, the exploratory data analysis of principal component analysis (PCA) was used. The main output of PCA is the loading plot and score plot. Samples with similar score plots had similar characteristics according to the variables used during PCA, therefore, the score plot of the first principle component (PC1) and second principle component (PC2) is also known as latent variables (Zhang *et al*., 2017; Rivera-Pérez *et al*., 2022). Figure 2 reveals the score plot of PC1 and PC2 indicating that Sidaguri samples harvested at the same time are clustered in one group. From this result, it can be observed that there are no outlier samples in PCA results.

Table 1. The antioxidant activities of Sidaguri powder as evaluated by radical scavenging activities using DPPH radical assay and TEAC, TPC and TFC.

Sample	DPPH assay (IC_{50}) (mg/mL)	ABTS assay (TEAC) (mg TE/g Sidaguri powder)	TFC (mg QE/g Sidaguri powder)	TPC (mg EAG/g Sidaguri powder)
Sidaguri. P. 3.1	0.789	13.515	8.083	30.384
Sidaguri. P. 3.2	0.787	13.450	8.048	30.358
Sidaguri. P. 3.3	0.791	13.401	8.077	30.247
Sidaguri. P. 4.1	0.916	12.197	7.450	27.845
Sidaguri. P. 4.2	0.915	12.243	7.432	27.689
Sidaguri. P. 4.3	0.913	12.232	7.459	27.708
Sidaguri. P. 5.1	5.398	4.565	5.181	10.993
Sidaguri. P. 5.2	5.410	4.598	5.158	10.947
Sidaguri. P. 5.3	5.355	4.611	5.176	11.022

P. 3, P. 4 and P. 5 are Sidaguri samples taken from different harvesting times (third, fourth, and fifth).

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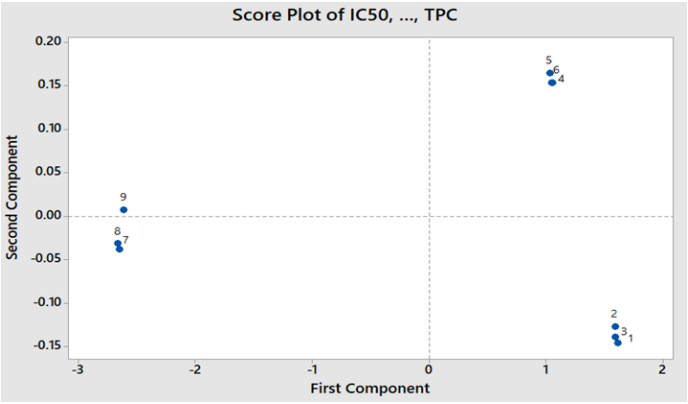

Figure 2. The score plot of the first principle component (PC1) and second principle component (PC2) for clustering of Sidaguri from different harvesting times. 1, 2, 3 = Sidaguri samples harvested at the third month; 4, 5, 6 = Sidaguri samples harvested at the fourth month; and 7, 8, 9 = Sidaguri samples harvested at the fifth month.

3.2 Prediction of antioxidant activities using FTIR and chemometrics

The antioxidant activities of Sidaguri samples were predicted using FTIR spectroscopy in combination with multivariate calibration. Figure 3 exhibits FTIR spectra of Sidaguri powder scanned at wavenumbers of the mid-IR region of 4000–650 cm^{-1}. The functional groups present in Sidaguri samples were represented by each peak and shoulder in FTIR spectra, in which the functional groups are responsible for infrared absorption. The peak at 3283 cm^{-1} was due to the stretching vibration of hydrogen bonding, the peak at 2920 cm^{-1} originates from C–H alkane stretch, while C–H aldehyde stretch was observed at wavenumbers of 2850 cm^{-1}, peak at 1732 cm^{-1} corresponded to the functional group of C=O carbonyls. The other assignment of functional groups can be seen in [24]. The pattern of FTIR spectra of the Sidaguri powders from different harvesting times is quite similar to each other. Therefore, it provided information on similar metabolites contained in the samples being assessed. The number of peaks, especially in fingerprinting regions, in FTIR spectra could be exploited as variables during the prediction of antioxidant activities of Sidaguri samples. Table 2 compiled the chemometrics results of multivariate calibrations of partial least square (PLS) using variables of absorbance values at different wavenumbers regions along with its spectral modes. In initial optimization, principle component regression (PCR) was also evaluated, however, the performance of PCR is lower than PLS, therefore PLS was further optimized. The selection of FTIR spectral condition-PLS relied on its performance to provide the highest R^2 values and lowest RMSEC and RMSEP values by considering the over-fitting phenomenon. Finally, absorbance values using normal spectra were selected. Figure 4A revealed the correlation between actual values of radical scavenging activities and predicted values based on FTIR spectra-PLS. The accuracy of the method was acceptable as indicated by high R^2-values (0.9995 and 0.9998 in calibration and validation models) with low errors, namely RMSEC of 0.0674 and RMSEP of 0.0827. In addition, the over-fitting does not occur because the residual analysis revealed that the error is located around zero (Figure 4B).

4. Conclusion

The harvesting time of Sidaguri revealed the effect of its antioxidant activities. Sidaguri harvested at a longer harvesting time tends to lower the antioxidant activities which can be correlated with the lower bioactive components responsible for the antioxidant activities. Furthermore, FTIR spectroscopy in combination with multivariate calibration of Partial least squares using absorbances at wavenumbers of 3700-650 cm^{-1} could provide the acceptable correlation modelling between actual values of antioxidant and predicted values with R^2-calibration and R^2-validation of > 0.999. The chemometrics of principal component analysis (PCA) could separate Sidaguri samples harvested during different months. FTIR spectra in combination with

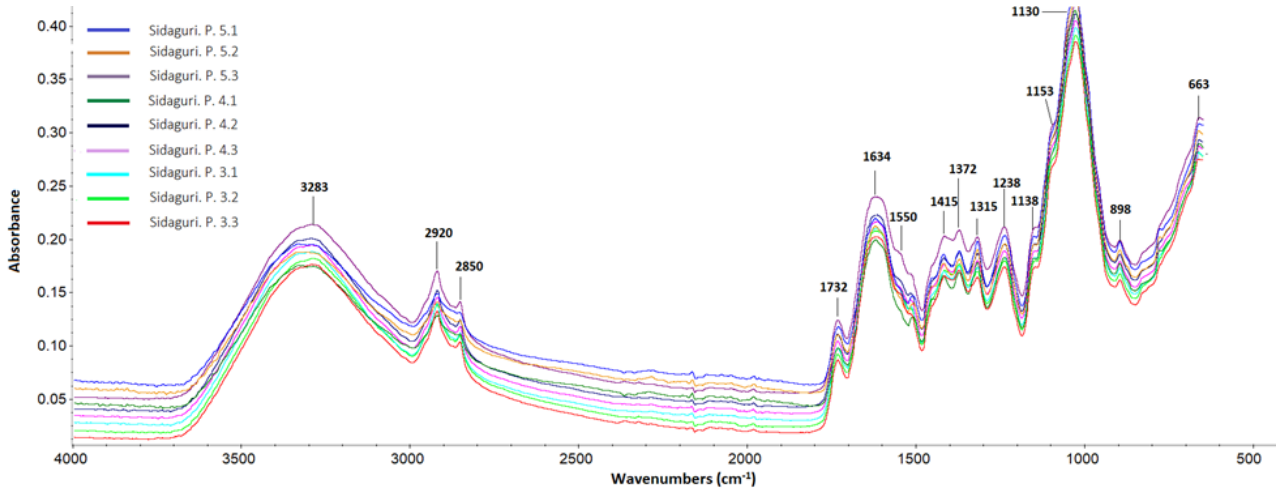

Figure 3. FTIR spectra of Sidaguri powder from different harvesting times scanned using attenuated total reflectance at wavenumbers of 4000–650 cm^{-1}.

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Table 2. Optimization results of antioxidant activity prediction using PCR and PLS.

Wavenumber (cm^{-1})	Multivariate calibration	Spectra	Calibration		Validation	
			R^2	RMSEC	R^2	RMSEP
3700-650	PLS	Normal	1.0000	0.0123	0.9998	0.0838
		Derivative 1	0.9995	0.0674	0.9998	0.0827
		Derivative 2	0.9999	0.0149	0.9999	0.1370
	PCR	Normal	0.9997	0.0500	0.9989	0.1040
		Derivative 1	0.9993	0.0789	0.9998	0.0829
		Derivative 2	0.9989	0.1010	0.9999	0.0755
3700-2800	PLS	Normal	0.9996	0.0626	0.9986	0.1180
		Derivative 1	0.9996	0.0573	0.9999	0.0525
		Derivative 2	0.9998	0.0429	0.9988	0.1140
	PCR	Normal	0.9996	0.0629	0.9986	0.1180
		Derivative 1	0.9994	0.0762	1.0000	0.0610
		Derivative 2	0.9996	0.0633	0.9987	0.1140
1800-700	PLS	Normal	1.0000	0.0007	0.9998	0.0918
		Derivative 1	1.0000	0.0125	0.9993	0.1080
		Derivative 2	0.9994	0.0736	0.9997	0.1080
	PCR	Normal	0.9988	0.1030	0.9971	0.1680
		Derivative 1	0.9992	0.0880	0.9986	0.1290
		Derivative 2	0.9992	0.0861	0.9997	0.1090

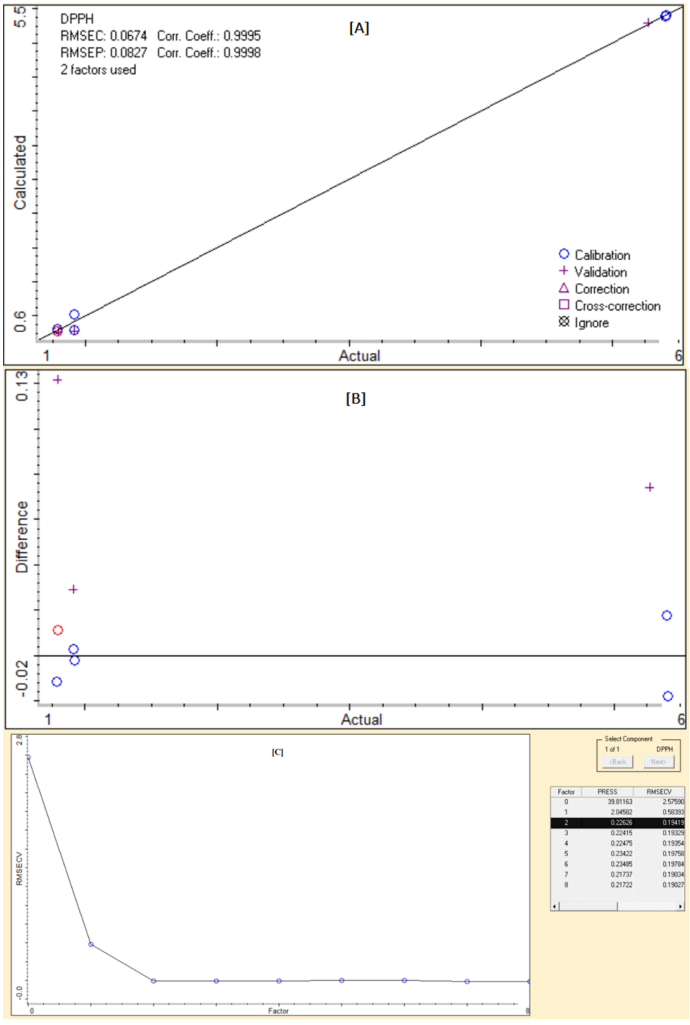

Figure 4. The correlation between actual values (x-axis) and FTIR predicted values (y-axis) of antioxidant activity of (A) Sidaguri, (B) residual factor, and (C) PRESS and RMSECV.

chemometrics could be an alternative method for the prediction of antioxidant activities of Sidaguri with acceptable accuracy and precision.

Conflict of interest

The authors declare no conflict of interest.

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