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FTIR-based Fingerprinting and Chemometrics for Rapid Investigation of Antioxidant Activity from *Syzygium polyanthum* Extracts

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Abstract: Syzygium polyanthum, *known as* salam *in Indonesia, which is rich in phenolics compounds with high antioxidant activity. In this study, we performed the determination of total phenolics and characterization of the antioxidant activity of* S. polyanthum *leaves extracts using the FTIR-based fingerprinting technique. The extracts of* S. polyanthum *in water, ethanol, and ethanol-water (30, 50, and 70%) were measured for their antioxidant activity, total phenolics, and FTIR spectra. The antioxidant activity of* S. polyanthum *extract with different solvent extraction showed the highest antioxidant activity and total phenolic content is 70% and 50% ethanolic extract, respectively. The FTIR spectrum of each extract showed identical FTIR spectra patterns. According to their different solvent extraction,* S. polyanthum *extract could be grouped based on FTIR spectra using principal component analysis. Correlation between the functional group in the FTIR spectra with IC$_{50}$ from* S. polyanthum *extract was analyzed using partial least square (PLS). The PLS analysis results showed that O–H, C–H sp^3, C=O, C=C, C-O, and C–H aromatic are the main functional groups contributed to the antioxidant activity of* S. polyanthum *extract. FTIR-based fingerprinting combined with chemometrics could be used to classify different extracts of* S. polyanthum *and predicted functional groups having a significant contribution to antioxidant activity.*

Keywords: *antioxidant; chemometrics; fingerprinting; FTIR;* S. polyanthum

■ INTRODUCTION

Syzygium polyanthum (*S. polyanthum*), or locally known in Indonesia as *salam*, belongs to the Myrtaceae family. Traditionally, *S. polyanthum* leaves were used as a food flavoring and medicinal uses. *S. polyanthum* extract known to have some biological activities such as antioxidant [1], antidiarrheal [2], antihypertensive [3], antihyperglycemic [4], antibacterial [5], cytotoxic and antiproliferative effect [6]. In addition, *S. polyanthum* extract could be used as a preservative for chicken meat [7]. Darusman et al. [1] reported that *S. polyanthum* extract showed inhibition of acetylcholinesterase, which is used for the treatment of Alzheimer's disease. Those biological activities came from *S. polyanthum* is related to the bioactive compounds found in this plant.

Chemical compounds present in *S. polyanthum*, such as flavonoids, alkaloids, steroids, triterpenoids, and tannins with the major class was from the flavonoids group [6]. Flavonoids are widely distributed in plants and are known to have various biological activities, and one of them is antioxidant activity [8]. The content of its bioactive compounds determines the characteristics of biological activity in plants. The method and extracting solvent type have a significant role in the extraction of metabolites because the content of various compounds in plants has different chemical characteristics and polarity [9]. Polar solvents such as water, ethanol, and a mixture of water and ethanol are widely used to extract phenolic compounds like flavonoids. Water and ethanol solvents have been reported to be effective in extracting

plant metabolites with intense biological activity [9-10]. Therefore, in this research, we used maceration with ethanol, water, and their mixture to analyze the effect of extracting solvent on extracted metabolites. In addition, the correlation of extracted metabolites showed in FTIR spectra with antioxidant activity in *S. polyanthum* leaves was determined.

The correlation between the composition of metabolites and its bioactivity of the plant extracts could be performed by the metabolomics approach. Metabolomics is the process of analyzing the metabolites present in organism comprehensively both quantitatively and qualitatively using multivariate analysis [11]. Metabolomics approach could display the profile of detected metabolites and evaluate the changes in the metabolite profile under various circumstances [12]. Finding the correlation of biological activity with their respective signal output of metabolites from spectrum or chromatogram is one example of the used metabolomics [13-14].

There are several papers reported in the study of metabolite profile changes and their correlation with bioactivity in plants by a combination of Fourier transform infrared (FTIR) spectra and chemometrics. This approach has been used for the correlation of metabolites and bioactivity to identify the functional group, which is correlated with antioxidant activity and inhibition of α-glucosidase of the crude extracts and fractions of *Phaleria macrocarpa*, *Momordica charantia*, and *Smallanthus sonchifolius* [15-17]. However, there is no reported study for the classification of *S. polyanthum* extracts using FTIR and its correlation with its antioxidant activity.

FTIR spectroscopy was chosen because it is a low-cost, quick, easy, and reproducible analysis. Hence, FTIR could be used as a method in classifying samples based on their biological properties. The FTIR spectrum produces complex data and difficult for interpreting the data, thus chemometrics assistance is needed to process the data. Chemometrics analysis used in this study were principal component analysis (PCA) and partial least square regression (PLS). PCA was used for classifying the samples according to its solvent extraction, while PLS was used for determining functional groups that give a

significant contribution to the antioxidant activity of *S. polyanthum*. In this work, we would like to classify *S. polyanthum* extracts with different solvent extraction and determined the functional group of compounds gives a significant contribution to the antioxidant activity of *S. polyanthum* extracts based on FTIR spectra.

■ EXPERIMENTAL SECTION

Materials

S. polyanthum leaves were obtained from Tropical Biopharmaca Research Center (TropBRC) medicinal plant garden, IPB University, Bogor, Indonesia. Sample identification was made by Mr. Taopik Ridwan (TropBRC), and a voucher specimen was stored in TropBRC, IPB University, Bogor, Indonesia. Ethanol absolute was purchased from Merck (Darmstadt, Germany). Ascorbic acid, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, potassium bromide for IR spectra, and sodium carbonate were obtained from Sigma Aldrich (Steinheim, Germany).

Instrumentation

Rotary evaporator-114 (Buchi, Flawil, Switzerland) was used for the evaporation of solvent extraction. Measurement of total phenolic and antioxidant activity was performed in Epoch microplate reader (BioTek Instruments Inc. Winooski, USA). FTIR spectra were measured in Bruker Tensor 37 FTIR spectrophotometer (Bruker Optik GmbH, Karlsruhe, Germany).

Procedure

Sample preparation and extraction

Fresh *S. polyanthum* leaves were cleaned with water and dried in the oven at 45 °C. After that, the samples were pulverized to obtain 40 mesh powder samples. The extraction of the samples was carried out by maceration according to the method developed by BPOM [18] with slight modification. Different solvent extraction, i.e., ethanol, 30% ethanol, 50% ethanol, 70% ethanol, and water, were used for extraction of the sample. A total of 10 g of *S. polyanthum* leaves powder and 100 mL of solvent were put into the Erlenmeyer and soaked for 6 h while stirring occasionally. The sample

was immersed for 3 × 24 h in a dark room. Furthermore, the filtrate was collected by filtering the mixture using filter paper. Each sample extract was concentrated using a rotary evaporator. The sample extract is then ready to be used for further analysis.

Determination of total phenolic

Total phenolic content was determined using the Folin-Ciocalteu method using the procedure of Prekumara et al. [19] in 96 well microplates. Sample extracts were weighed about 10 mg and dissolved in 1 mL of ethanol, obtaining an extract solution with a concentration of 10,000 μg/mL. The extract solution was diluted to 250 μg/mL. About 20 μL of extract solution was mixed with 110 μL of Folin-Ciocalteu reagent and 70 μL of sodium carbonate and incubated at 25 (± 2) °C for 30 min. After that, the absorbance of the solution was measured at 765 nm. Total phenolic content was expressed as gallic acid equivalent (GAE) in grams of dried powder sample (mg GAE/g dried powder).

Determination of antioxidant activity

The determination of antioxidant activity was performed by the DPPH method using the procedure developed by Salazar et al. [20]. A total of 100 μL of *S. polyanthum* extract and 100 μL of DPPH 125 μM were mixed in the 96 well microplates, then incubated at room temperature for 30 min. The absorbance of the solution was measured at 517 nm against ethanol as a blank. The concentration range used was between 1.56 to 25.00 μg/mL for *S. polyanthum* extract solution. Ascorbic acid was used as a positive control, and the antioxidant activity was reported as IC_{50} that can be calculated by the correlation of each concentration with the inhibition value of DPPH.

FTIR spectra

FTIR spectra were measured in the FTIR spectrophotometer Tensor 37 (Bruker, Ettlingen, Germany) equipped with a deuterated triglycine sulfate detector. The sample was prepared by mixing 2 mg of extract and 180 mg of KBr until homogeneous, and then the pellet was formed with a pressure of 8 tons for 15 min using a manual compression. The pellet was placed in the sample compartment. Measurement of the FTIR spectra was performed in the region of 4000–400 cm^{-1} with a resolution of 4 cm^{-1} and 32 scans/min controlled by OPUS 4.2 software (Bruker, Ettlingen, Germany). FTIR spectra were stored as a data point table.

Chemometrics analysis

Analysis of variance (ANOVA) and Duncan test were used to determine the significant difference between the results of extraction yield, total phenolics content, and antioxidant activity. A significant difference was defined at the 95% confidence level ($p < 0.05$). Classification of *S. polyanthum* extracts according to the solvent extraction was performed using PCA. The identification of functional groups in antioxidant compounds was determined using PLS analysis by a correlation between the FTIR spectra and IC_{50} from antioxidant activity. The Unscrambler X version 10.1 (CAMO, Oslo, Norway) was used for running the PCA and PLS analysis.

■ RESULTS AND DISCUSSION

Extraction Yield and Total Phenolic Content

S. polyanthum leaves were extracted by maceration using ethanol, water, and their mixture as solvent extraction. Solvent replacement after 24 h for three times aims to extract more effectively and gave an increase in the yield. Extraction with 30% ethanol gave the highest yield followed by 50% ethanol, 70% ethanol, ethanol, and water (Table 1), indicating the different polarity of the solvent extraction give a different level of extracted metabolites.

The determination of phenolic content in *S. polyanthum* leaves extract was performed using the Folin-Ciocalteu method. In this method, the oxidation reaction of phenolic compounds in an alkaline condition by the Folin-Ciocalteu reagent will produce a blue molybdenum-tungsten complex that provides strong absorption at a wavelength of 750 nm. Total phenolic content is reported as the gallic acid equivalent. The results of total phenolic content in *S. polyanthum* extract are shown in Table 1.

Total phenolic levels in *S. polyanthum* extracts in the order from the highest to the lowest are 50% ethanol > 30% ethanol > 70% ethanol > ethanol > water. The

Table 1. Extraction yield, total phenolic content, and antioxidant activity of *S. polyanthum* extracts

Solvent extraction	Extraction yield (%)	Total Phenolic content (mg GAE/g dried powder)	Antioxidant activity (IC$_{50}$, μg/mL)
Ethanol	12.46 ± 0.95^a	2.21 ± 0.24^a	6.75 ± 0.35^a
30% ethanol	27.42 ± 4.54^b	6.87 ± 0.83^c	8.92 ± 0.51^a
50% ethanol	25.94 ± 0.98^b	6.88 ± 0.80^c	7.59 ± 0.16^a
70% ethanol	22.46 ± 0.42^b	4.66 ± 0.58^b	6.19 ± 0.78^a
Water	11.42 ± 3.93^a	1.90 ± 0.68^a	21.13 ± 4.38^b

The reported values are mean ± SD of the triplicate assay for each sample. The mean ± SD within each extract in the same column followed with different superscript letters represent significant differences at $p < 0.05$

results of total phenolic content showed that phenolic could be extracted higher in mixed ethanol-water solvents compared to the pure solvents. The most efficient extracting solvent is 30%, and 50% ethanol for extracting phenolic compounds in *S. polyanthum* leaves with total phenolic content about 6.87 and 6.88 mg GAE/g dried powder, respectively. The two extracts gave higher results compared to other extracts and not significantly different in total phenolic content as a result of ANOVA.

Antioxidant Activity of *S. polyanthum* Extracts

The antioxidant activity of the sample was determined by the DPPH method. The DPPH method has the advantages, such as easy to perform, quick analysis, and provide the information on the reactivity of compounds tested using a stable radical. DPPH solution when mixed with a substance that can donate hydrogen atoms with a change in color from purple to yellow [21]. The higher the antioxidant activity of a sample, the lower the intensity of the remaining purple color will be, so that its absorption at a wavelength of 517 nm will be smaller. Increasing the concentration of the sample would give a higher percentage of DPPH inhibition, meaning the higher antioxidant activity that can inhibit more free radicals. Based on the measurement results obtained, the value of absorbance decreased with increasing concentrations of *S. polyanthum* leaves extract.

The antioxidant activity of each extract was expressed with IC$_{50}$ value. The IC$_{50}$ values were obtained from the equation in the correlation curve of *S. polyanthum* extract concentration and the percentage of DPPH inhibition. According to Molyneux [21], a sample with IC$_{50}$ < 50 μg/mL, between 50–100 μg/mL, and > 200 μg/mL

is classified as strong, weak, and very weak antioxidant activity, respectively. The antioxidant activity of the five *S. polyanthum* leaves extracts is classified as strong because it has an IC$_{50}$ value below 50 μg/mL.

From the result obtained, 70% ethanol extract has the smallest IC$_{50}$ followed by ethanol, 50% ethanol, 30% ethanol, and water (Table 1). So, 70% ethanol extract showed the highest antioxidant activity. As can be seen, when the water ratio decreases, the antioxidant activity significantly increases. However, when ethanol only was used, the antioxidant activity did not give higher results compared to 70% ethanol extract. Mediani et al. [22] also found that extraction with ethanol only gave lower antioxidant activity compared to the 70% ethanol extract in *Cosmos caudatus*.

Classification of *S. polyanthum* Leaves Extracts Based on IR Spectra and Chemometrics

Fig. 1 shows the IR spectra of each *S. polyanthum* leaves extract. From the IR spectra, no differences are obtained in the peak position, or each extract has a similar pattern and only differ in their intensities. Water extracts showed a lower peak absorption intensity compared to the other extracts. Meanwhile, very similar patterns and intensities are found in 30% and 50% ethanol extracts. These similar patterns in each extract mean that there is a similar composition in the metabolite extracted from *S. polyanthum* leaves using each solvent extraction. Therefore, the classification of *S. polyanthum* leaves extracts by using only IR spectra will not be easy, so that aid from chemometrics analysis is needed to discriminate each extract.
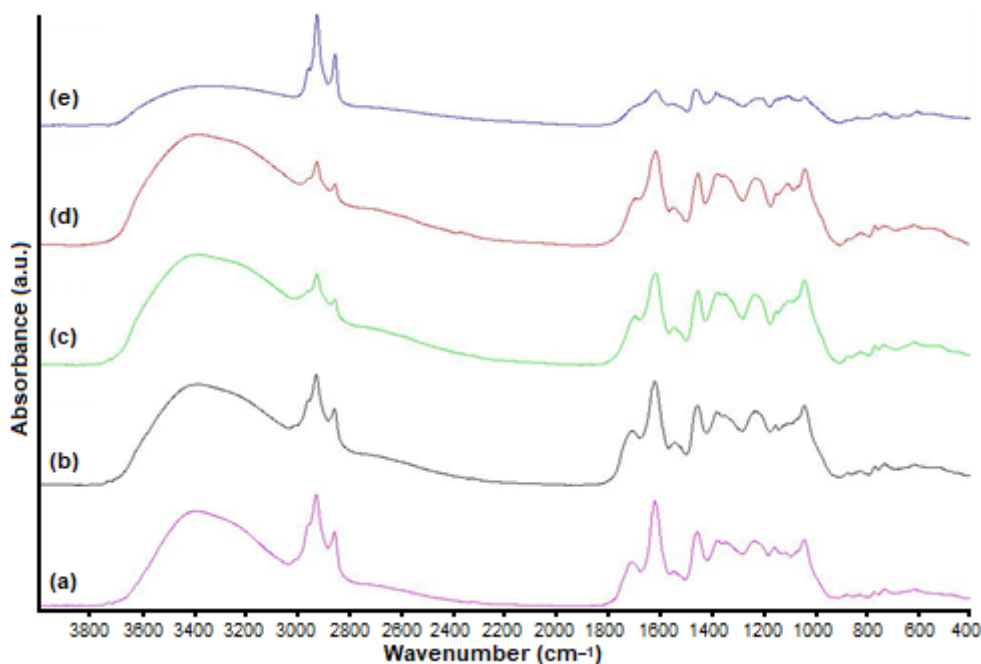
The identification of the functional groups found in

Fig 1. FTIR spectrum of *S. polyanthum* (a) EtOH, (b) 70% EtOH, (c) 50% EtOH, (d) 30% EtOH, and (e) water extract

the *S. polyanthum* leaves extracts is shown in Table 2. There is a difference in the intensity of the absorbance value at a wavenumber of 1680–1600 cm^{-1} with the highest value found in 70% ethanol and the lowest value found in the water extract. At the wavenumber of 900–690 cm^{-1}, the water extraction absorption has a lower value than other extracts. The differences in the absorbance values at some peaks in each extract indicates differences in the amount of metabolite.

In this study, PCA was used for the classification of *S. polyanthum* extracts according to different solvent extraction. Before subjected to PCA, each spectrum was pre-processed to reduce variations in the data but did not have any effect on the analytical information. PCA worked to simplify the observed variables by reducing their dimensions, and with the score plot obtained from the principal component, a grouping sample was obtained. Fig. 2 shows the PCA score plot of the *S. polyanthum* leaves extracts.

In the PCA score plot, all of the extracts were classified into their group (five classes). The first two PC's were used with 88% total variance with PC-1 explained 59%, and PC-2 explained 29% of the variance. In Fig. 2, the 30% ethanol and 50% ethanol extract were adjacent for

their position in the PCA plot. This closeness shows that the composition and level of metabolite extracted with those solvents are almost similar.

Correlation between *S. polyanthum* Leaves Extract FTIR Spectra with Antioxidant Activity

Identification of functional group, which has a major contribution to the level of antioxidant activity of *S. polyanthum* leaves extracts, was determined using the partial least square (PLS) regression method. In the PLS modeling, it should have a variable data and response variables. Antioxidant activity, expressed as IC$_{50}$ obtained by the DPPH method, was used as the response variable (y), while the absorbance from FTIR spectra was used as the estimating variable (x). Before subjected to the

Table 2. Functional group present in *S. polyanthum* extracts

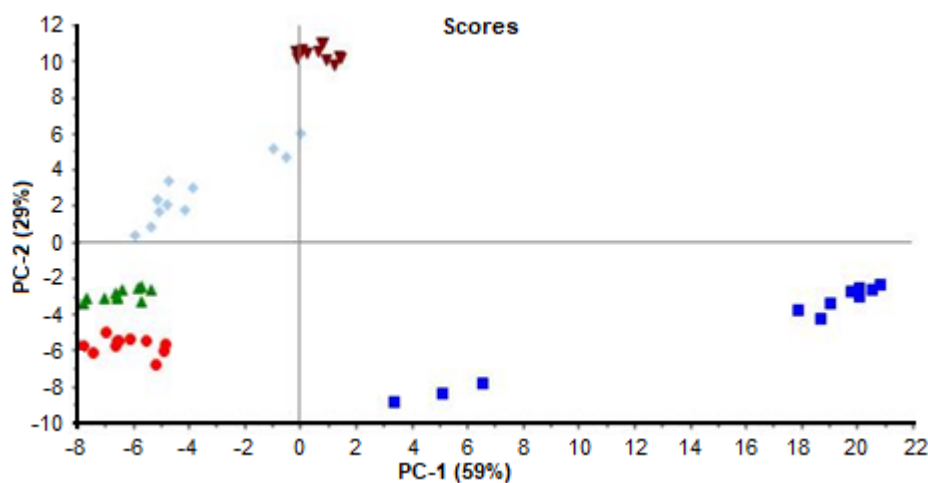| Wavenumber (cm^{-1})	Assign functional group
3400–3300	O–H
3000–2840	C–H sp^3
1740–1720	C=O
1680–1600	C=C
1300–1000	C–O
900–690	C–H aromatic

Fig 2. PCA plot of *S. polyanthum* leaves (▼) EtOH, (♦) 70% EtOH, (▲) 50% EtOH, (●) 30% EtOH, and (▪) water extract

PLS, the FTIR spectra were given the same pre-processing treatment as in the PCA.

The X-Y relation score and regression coefficient plot will be obtained in the PLS regression. The X-Y relation score plot was shown in Fig. 3. This plot explains the relationship between the absorbance data related to the functional groups as variable X and antioxidant activity as variable Y. Also, the plot shows that a grouping of extracts exhibit active and not active as antioxidant sources. An active extract with high antioxidant activity is grouped in the negative area. In contrast, the less active extracts are in the positive area of the plot because the low IC_{50} value indicates a high antioxidant activity. As can be seen in Fig. 3, 30% ethanol, 50% ethanol, 70% ethanol, and

ethanol extracts were the active extract because the four extracts had lower IC_{50} values compared to water extracts.

In this study, the identification of a functional group from a metabolite that gives a major contribution to the antioxidant activity could be identified from the plot of the regression coefficient in the PLS analysis. The plot of regression coefficients (Fig. 4) is used to summarize the relationship between all predictors and responses given to PLS; the regression coefficient can take into account several components or factors. For example, in Fig. 4, the model is obtained from factor 5. It has information that the regression coefficient can summarize the relationship between the predictor and
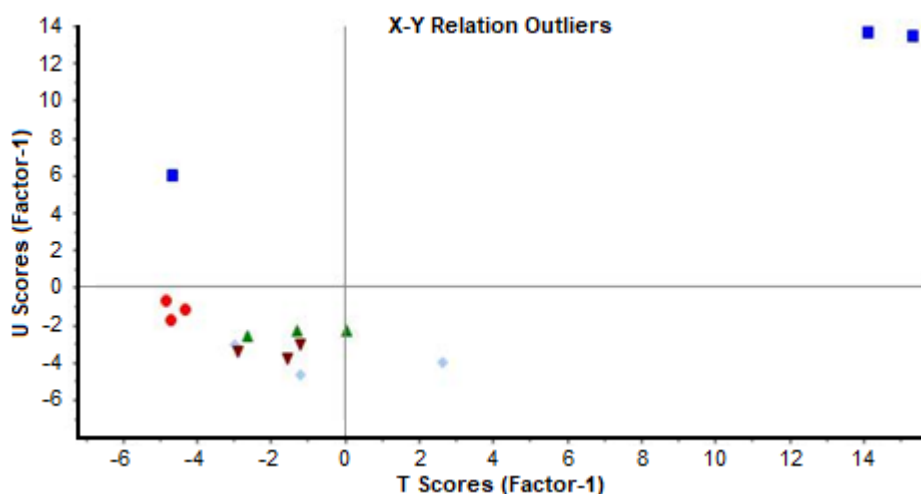

Fig 3. PLS plot as a relationship between absorbance from the functional groups and antioxidant activity of *S. polyanthum* leaves (▼) EtOH, (♦) 70% EtOH, (▲) 50% EtOH, (●) 30% EtOH, and (▪) water extract
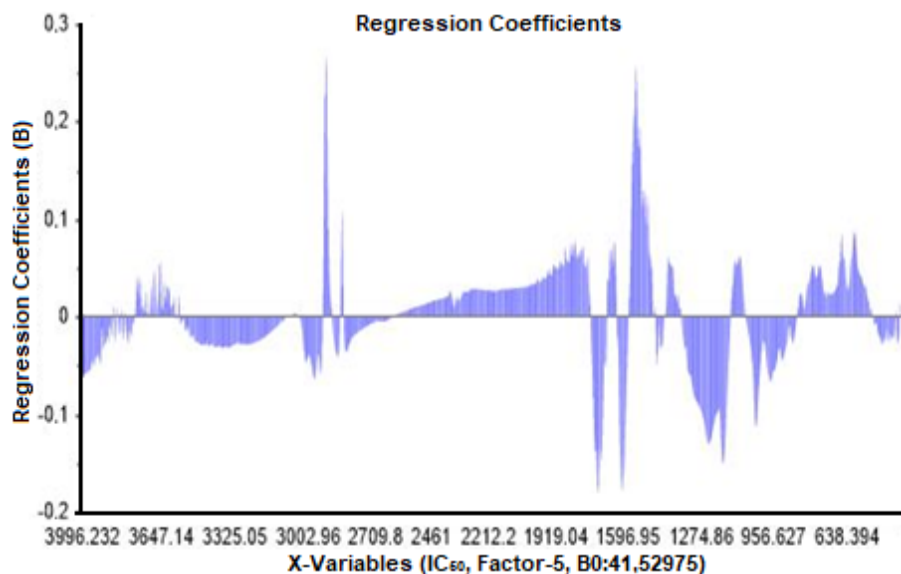
Fig 4. Regression coefficient plot of *S. polyanthum* extracts
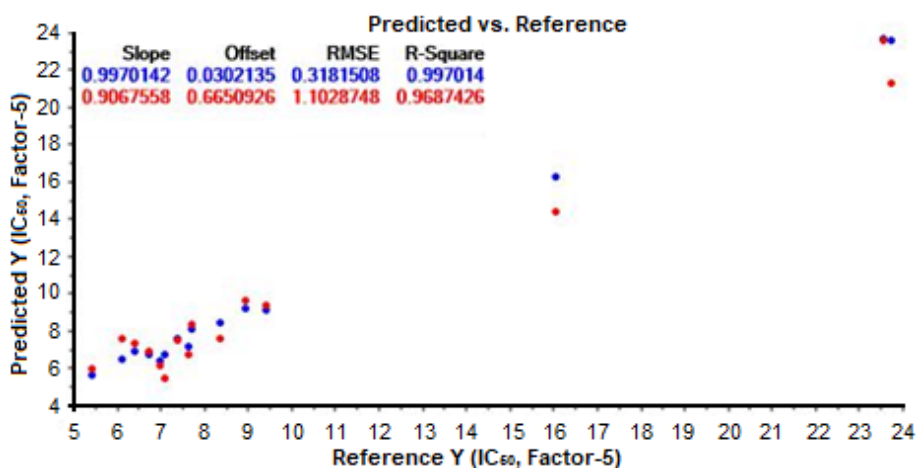

Fig 5. Predicted vs. reference plot for calibration and validation *S. polyanthum* extracts

response by using factor 5 as the best model predicted. Plot regression coefficients provide information about how important the variable X to variable Y. Variable X, which has a large regression coefficient, is a variable that has an important role in the regression model. The absorption of functional groups that play an active role in antioxidant activity expressed by IC_{50} values has a negative regression coefficient [14]. Fig. 4 shows that there are several functional groups have a negative regression coefficient, such as O–H (3400–3080 cm⁻¹), C–H sp³ (3012–2831 cm⁻¹), C=O (1740–1720 cm⁻¹), C=C (around 1600 cm⁻¹), C–O (1300–1000 cm⁻¹), and C–H aromatics (900–690 cm⁻¹). Thus, the antioxidant

compound in the *S. polyanthum* would have those functional groups with C=O has the lowest regression coefficient value. From the result obtained, it could be predicted that the antioxidant compound from *S. polyanthum* leaves extract is mainly from phenolics class such as phenolic acid, flavonoids, etc.

Validation of the predictive model is very important to avoid overfitting in the model. The cross-validation method was used, and the result is showed as predicted vs. reference plot for calibration and validation (Fig. 5). In this plot, a good predictive model will obtain linearity (R^2) between the predicted value and the measured value is close to 1, and the root mean

square error (RMSE) for calibration and validation is close to 0. As can be seen from the plot, the R^2 for calibration and validation were 0.977 and 0.969, while the RMSE for calibration and validation were 0.32 and 1.10, respectively. Hence, in this study, the PLS model was categorized as a good model because the R^2 and RMSE value is close to 1 and 0, respectively.

■ CONCLUSION

Antioxidant activity and FTIR spectrum of *S. polyanthum* leave extract are affected by variations in solvent concentration. The highest antioxidant activity and total phenolic content were shown in 30% and 50% ethanol extract, respectively. FTIR spectra of each extract have a similar pattern but could be grouped by PCA with a total variance of PC-1 and PC-2 about 88%. Based on PLS analysis, it was found that O–H, C–H sp^3, C=O, C=C, C–O, and C–H aromatic give a contribution to the antioxidant activity of *S. polyanthum* leave extract.

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