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The employment of UV-Vis spectroscopy and chemometrics techniques for analyzing the combination of genistein and curcumin

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ABSTRACT

In recent years, the combination of genistein (GEN) and curcumin (CUR) has attracted much attention due to its potential activity as an immunomodulatory agent. However, it is important for ensuring the quality and safety of the products using a simple, rapid, and cost-effective analytical method. UV-Vis spectroscopy method in combination with chemometrics techniques was successfully exploited to analyze the combination of GEN and CUR. Multivariate calibration models, namely, principal component regression (PCR) and partial least squares regression, were generated in this study using the R statistical software. It was found that the best predictive models for the quantitative determination of GEN and CUR were PCR on Savitzky-Golay smoothing spectral and PCR of original spectra, respectively. The sparse partial least square-discriminant analysis model was built with parameter tuning. The classification of GEN, CUR, their binary mixture, and methanol was successfully conducted by tuning the model parameters, including the prediction distance, number of components, and the "keepX" variables.

INTRODUCTION

Two or more drugs can be combined to increase efficacy, decrease toxicity, and reduce drug resistance to implement a promising approach for obtaining better health treatment (Foucquier and Guedj, 2015). Genistein (GEN), an isoflavone aglycone from *Glycine max*, was reported due to its beneficial function not only for daily consumption but also for health enhancement (Sirotkin, 2014). Soy GEN increased human immunity and proved to be potential antibreast cancer and immunomodulatory agents for postmenopausal women (Ryan-Borchers *et al*., 2006; Sakai and Kogiso, 2008; Yuliani *et al*., 2016). Curcumin (CUR), a secondary metabolite contained in *Curcuma longa* L., was commonly consumed to increase human immunity (Catanzaro *et al*., 2018). A recent study reported the potential activity of CUR as a therapeutic agent against pneumonia and acute respiratory distress syndrome caused by virus infection (Liu and Ying, 2020). The molecular mechanism of combination flavonoid immunomodulatory agents was studied (Hosseinzade *et al*., 2019). Other recent studies demonstrated the synergistic effect of combination GEN and CUR as an antitrypanosomal agent (Ettari *et al*., 2019).

The increasing research interest on the combination of GEN and CUR should be supported with the development of an analytical method to ensure the quality and safety of the product. GEN and CUR were reported to have UV-Vis absorbance in the range of 200–800 (Kadam *et al*., 2018; Luan *et al*., 2017). However, the interference between GEN and CUR spectral profiles near 260 nm can become the limitation for performing simultaneous conventional UV-Vis method (Priyadarsini, 2014; Yatsu *et al*., 2016). Chromatographic techniques such as high-performance liquid chromatography (HPLC) and high-performance thin-layer chromatography (HPTLC) can be applied in the analysis of drugs, foods, and herbal products (Dwiastuti *et al*., 2018; Prabaningdyah *et al*., 2017; Riswanto *et al*., 2015; Satpathy *et al*., 2017; Yuliani *et al*., 2018). However, chromatographic methods were quite expensive and time-consuming compared to the spectroscopic method (Suhandy and Yulia, 2017).

The aim of this study was to combine UV-Vis spectroscopy with chemometrics techniques for analyzing GEN and CUR simultaneously without any separation stages.

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Spectral data of UV-Vis can be processed using an appropriate chemometrics algorithm for both quantitative and qualitative analyses (Hussain *et al*., 2018; Kambira *et al*., 2020). Multivariate calibration of PCR and partial least squares (PLS) regression was generated to quantitatively analyze the content of GEN and CUR in the binary mixture. Sparse partial least square-discriminant analysis (sPLS-DA), a supervised pattern recognition model, was built to differentiate between GEN, CUR, the mixture of GEN and CUR, and its solvent.

MATERIALS AND METHODS

Materials and instrumentation

GEN and CUR standards were purchased from Sigma Aldrich. The solvent used in this study was methanol (BLANK) obtained from Merckmillipore. A system of UV-Vis Spectrophotometer type UV 1,800 (Shimadzu, Japan) equipped with quartz cuvette 1 cm (Hellma, Plainview, NY) was controlled using UVProbe Software (Shimadzu, Japan) to scan and record all UV-Vis spectra of samples. An ultra-micro-analytical balance RADWAG® series of UYA 2.3Y (max. 2.0 g, minute 0.01 mg, and readability 0.1 µg) was utilized in this study. A set of Socorex® micropipettes was used in preparing the solutions. The data of UV spectral were exported to Excel 2016 (Microsoft Inc., Redmond, WA) and saved in the format files of .csv.

Chemometrics software

Multivariate calibrations and spectral preprocessing were carried out using R Studio software version 1.1.456 with "pls" and "prospectr" packages, respectively (Mevik and Wehrens, 2007; Stevens *et al*., 2020). sPLS-DA modeling and tuning were generated using "mixOmics" package (Rohart *et al*., 2017).

Standard solutions preparation

Accurate weights of 9.585 mg of GEN and 9.755 mg of CUR were transferred into two separated 50 ml volumetric flasks for each compound, followed by dilution with BLANK into the volume. These solutions were labeled as GEN and CUR stock solution.

Preparation of calibration and validation standard solutions

A set of calibration and validation solutions containing GEN and CUR was prepared from the stock solutions to obtain 25 concentration variation of calibration solutions and 15 concentration variation of validation solutions as presented in Table 1. The concentration variation for both calibration and validation solutions was randomly assigned using Excel 2016 (Microsoft Inc., Redmond, WA).

Solutions for the sPLS-DA study were prepared in four classes as to which they would be categorized as follows: blank/solvent solution or BLANK, GEN solution, CUR solution, and binary mixture containing GEN and CUR. GEN solutions were prepared by transferring 400, 405, 410, and 415 µl of GEN stock solution into a 5 ml volumetric flask, followed by dilution with BLANK into the volume. CUR solutions were prepared by transferring 400, 405, 410, and 415 µl of CUR stock solution, followed by dilution with BLANK into the volume. Fifteen binary mixtures of GEN and CUR were obtained from the calibration and validation set solutions chosen randomly.

Spectroscopic analysis for generating multivariate calibration

All prepared solutions were scanned using UV-Vis Spectrophotometer type UV 1,800 (Shimadzu, Japan) at 200–800 nm with an interval of 2 nm. The absorbance values of every single wavelength point obtained from the scanning process were collected and statistically analyzed using the R studio software. Absorbance data of calibration and validation solution were treated and preprocessed into five types of UV-Vis spectral, namely, original, first derivative, second derivative, standard normal variate (SNV), and Savitzky-Golay (SG) smoothing with a window width of 11 points and polynomial order three.

Two multivariate calibration models of PCR and PLS were generated and applied in order to generate a suitable predictive model for each compound. Multivariate calibration

Table 1. Information on calibration and validation data sets for model selection and statistical analysis.

Items	Datasets	
	Calibration	Validation
Number of mixture standards	25	15
GEN concentration (µg/ml)		
Mean	11.77	13.62
Range	4.79–18.59	5.75–18.59
CUR concentration (µg/ml)		
Mean	12.18	12.40
Range	4.88–19.51	5.27–18.92
Multivariate calibration models	PCR	PCR
	PLS	PLS
Evaluated parameters for model selection	R_{cal}^2	R_{val}^2
	RMSEC	RMSEP
	RMSECV[a] and R_{CV}^2	

[a]Cross-validation was carried out using the leave-one-out technique.

model performance was evaluated by assessing several statistical parameters such as coefficient of determination for calibration (R_{cal}^2), cross-validation (R_{CV}^2), validation (R_{val}^2), root mean square error of calibration (RMSEC), root mean square error of cross validation (RMSECV), and root mean square error of prediction (RMSEP). In addition, cross-validation as internal validation was carried out using the leave-one-out technique. The selected multivariate calibration model for each compound was determined by evaluating values of R^2 and their root mean square error values.

Modeling and tuning of sPLS-DA model

GEN, CUR, their binary mixture, and BLANK as a solvent were discriminated statistically by employing sPLS-DA technique. The sPLS-DA classification model was visualized by 3D and background plot generated using the maximum prediction distance. Classification performance of generated sPLS-DA model was evaluated using the area under the receiver operating characteristic (AUROC). The sPLS-DA performance was determined as a classification error rate and a balanced error rate (BER). Analysis of classification error rate and BER resulted in the optimum number of components which obtained the best performance considering the misclassification error rate and BER. Hence, the final result of sPLS-DA was built by applying the optimum number of components to the model and visualized as an individual sPLS-DA plot.

RESULTS AND DISCUSSION

Spectroscopic analysis

The conventional spectroscopy technique was commonly utilized to quantitatively analyze a single component of analyte using one or two wavelength detection approaches (Edwards *et al*., 2001). However, determining multiple components in a mixture by multiple wavelength spectroscopic detection is now challenging due to the reality of broad range drug combination or other real samples (Dzulfianto *et al*., 2018; Suhandy and Yulia, 2017). GEN, one of the isoflavone aglycones, was characterized by the UV absorption at 260 nm due to the presence of chromophore and auxochromes of hydroxyl from its polyphenolic structure (Maskey *et al*., 2003; Riswanto *et al*., 2020; Yatsu *et al*., 2016). CUR showed identical absorption in the wavelength range from 350 to 450 nm and in the UV region range of 250–270 nm (Priyadarsini, 2014).

Figure 1 shows the UV spectral profiles and chemical structures of GEN, CUR, and their combination. GEN showed the maximum absorption at 260 nm, while CUR spectral was characterized by maximum absorption at 420 nm and the presence of a smaller absorption peak at 262 nm. Notably, the spectral overlapping at 260–262 nm contributed to the limitation of simultaneous analysis of UV-Vis spectroscopy technique. Fortunately, it is possible to employ the chemometrics technique such as multivariate calibration and discriminant analysis to overcome this problem. The simultaneous analysis of GEN and CUR can be carried out by the UV-Vis method using appropriate chemometrics and parameters selection techniques.

Multivariate calibration

Multivariate calibration was initially conducted by preprocessing raw data of scanned spectral into five types of UV-Vis spectral such as original, first derivative, second derivative, SNV, and SG. The purpose of spectral preprocessing was to improve the subsequent bilinear calibration model and make it possible to select a type of preprocessed spectra which resulted in

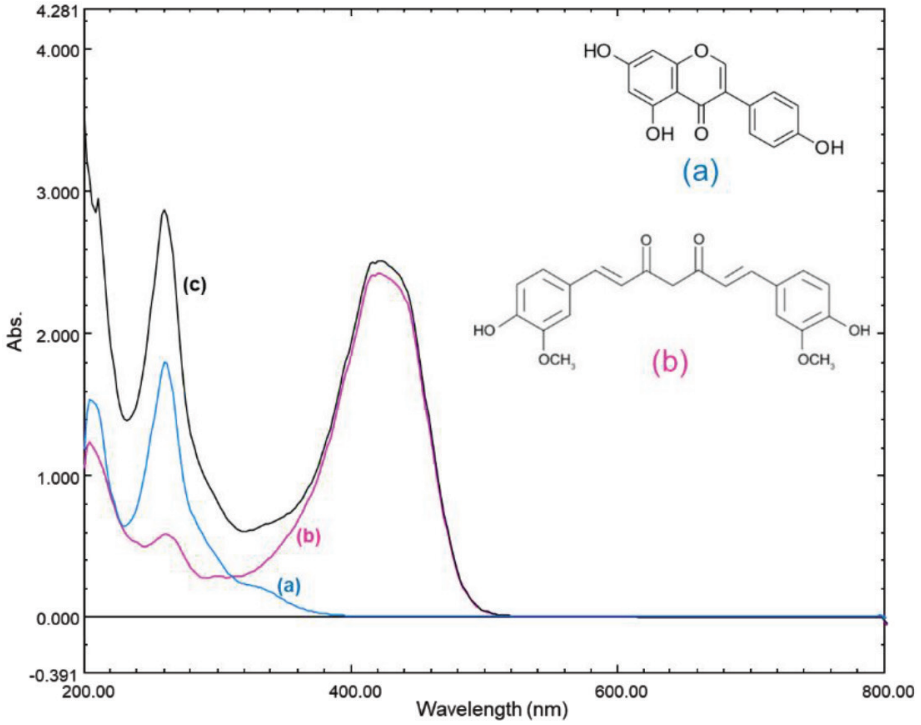

Figure 1. The UV-Vis spectral profiles and chemical structures of GEN (a), CUR (b), and their binary mixture (c).

the best multivariate calibration model (Rinnan *et al*., 2009). Two models of multivariate calibration, namely, PCR and PLS, were developed for all types of spectra. PLS regression was generated using a linear combination of the predictor variables rather than the original, while PCR was built to reduce the number of predictor variables by using their first few principal components (Miller and Miller, 2010).

Table 2 presents the statistical performance of PCR and PLS models for analyzing GEN and CUR simultaneously. PCR of SG spectra and PCR of original/normal spectra were selected as multivariate calibration models for GEN and CUR, respectively. Statistical parameters, namely, R^2 (R_{cal}^2, R_{CV}^2, and R_{val}^2) and RMSE (RMSEC, RMSECV, and RMSEP) were assessed for selecting the best predictive multivariate calibration model for each compound. The highest value of R^2 and the lowest value of RMSE were considered as selection criteria since the highest R^2 represents smaller differences between the actual and predicted values, while the lowest RMSE indicates better fit with minimum errors (Irnawati *et al*., 2020). Cross validation was conducted in this study using the leave-one-out technique in order to avoid the over-optimistic of prediction (Shen *et al*., 2018).

Figure 2 showing multivariate calibration properties of the selected model for GEN and CUR. The equation for correlating between the actual and the predicted values of GEN was $y = 1.0651x-0.853$ ($R^2 = 0.994$). The prediction plot of the GEN equation is shown in Figure 2a. The regression coefficient plot of the GEN model is shown in Figure 2b and provided useful information along with confidence intervals from different wavelengths (Jann, 2014). It should be noted that valleys and peaks at a certain wavelength are indicated to be important for GEN determination. The equation for correlating between the actual and the predicted values of CUR was $y = 0.969x + 0.498$ ($R^2 = 0.983$). The prediction plot of the CUR equation is shown in Figure 2c. The regression coefficient plot of the CUR model is shown in Figure 2d. The important wavelength for generating a multivariate calibration model of CUR was observed at the range of 350–500 nm indicated by the presence of an extensive peak at this region. Surprisingly, the important wavelengths for both GEN and CUR were found at a similar wavelength range compared to the maximum absorption wavelength from their original spectra. From the regression coefficient plots, it was confirmed that UV-Vis spectroscopy is a suitable analytical method for analyzing GEN and CUR. In addition, the visible spectrum range of >500 nm resulted in a very low contribution towards multivariate calibration modeling since there was no significant absorption in this region.

sPLS-DA modeling

In this study, sPLS-DA modeling has been developed. sPLS-DA, a variant of PLS-DA, provided useful advantages in high dimensional data analysis due to its ability to achieve variable selection and dimension reduction simultaneously (Chung and Keles, 2010; Ruiz-Perez and Narasimhan, 2018). Although the PLS algorithm was commonly designed to overcome regression problems, it can be employed as classifier tools in discrimination studies (Lê Cao *et al*., 2011).

Figure 3 shows the 3D visualization and background prediction of sPLS-DA performance generated using maximum

Table 2. Statistical performance of principal component regression (PCR) model and partial least squares (PLS) model for analyzing GEN and CUR.

Analytes	Model	Type of spectra	*N*comp	R_{cal}^2	RMSEC	R_{CV}^2	RMSECV	R_{val}^2	RMSEP
GEN	PCR	Original	6	0.992	0.393	0.982	0.573	0.993	0.292
		First derivative	6	0.989	0.479	0.979	0.618	0.983	0.47
		Second derivative	20	0.998	0.177	0.991	0.418	0.936	0.914
		SNV	9	0.968	0.805	0.922	1.204	0.903	1.124
		SG	**6**	**0.993**	**0.365**	**0.985**	**0.523**	**0.994**	**0.291**
	PLS	Original	3	0.989	0.4678	0.983	0.562	0.991	0.340
		First derivative	5	0.989	0.477	0.979	0.619	0.986	0.429
		Second derivative	12	0.999	0.117	0.983	0.562	0.920	1.020
		SNV	8	0.969	0.788	0.913	1.268	0.908	1.099
		SG	5	0.993	0.370	0.985	0.528	0.994	0.292
CUR	PCR	**Original**	**4**	**0.990**	**0.474**	**0.985**	**0.549**	**0.983**	**0.549**
		First derivative	7	0.992	0.439	0.981	0.633	0.975	0.669
		Second derivative	23	0.999	0.009	0.759	2.249	0.969	0.746
		SNV	8	0.979	0.699	0.943	1.093	0.953	0.914
		SG	2	0.989	0.494	0.986	0.535	0.981	0.586
	PLS	Original	3	0.990	0.469	0.985	0.554	0.982	0.571
		First derivative	5	0.991	0.453	0.982	0.617	0.975	0.670
		Second derivative	15	0.999	0.015	0.763	2.228	0.970	0.732
		SNV	8	0.979	0.685	0.931	1.196	0.956	0.891
		SG	2	0.989	0.494	0.986	0.535	0.981	0.587

Notes: Selected multivariate calibration model for genistein and curcumin were marked with bold. Ncomp: number of selected components; PCR: Principal Component Regression; PLS: Partial Least Squares; SNV: Standard Normal Variate; SG: Savitzky-Golay smoothing with window width of 11 points and polynomial order 3.

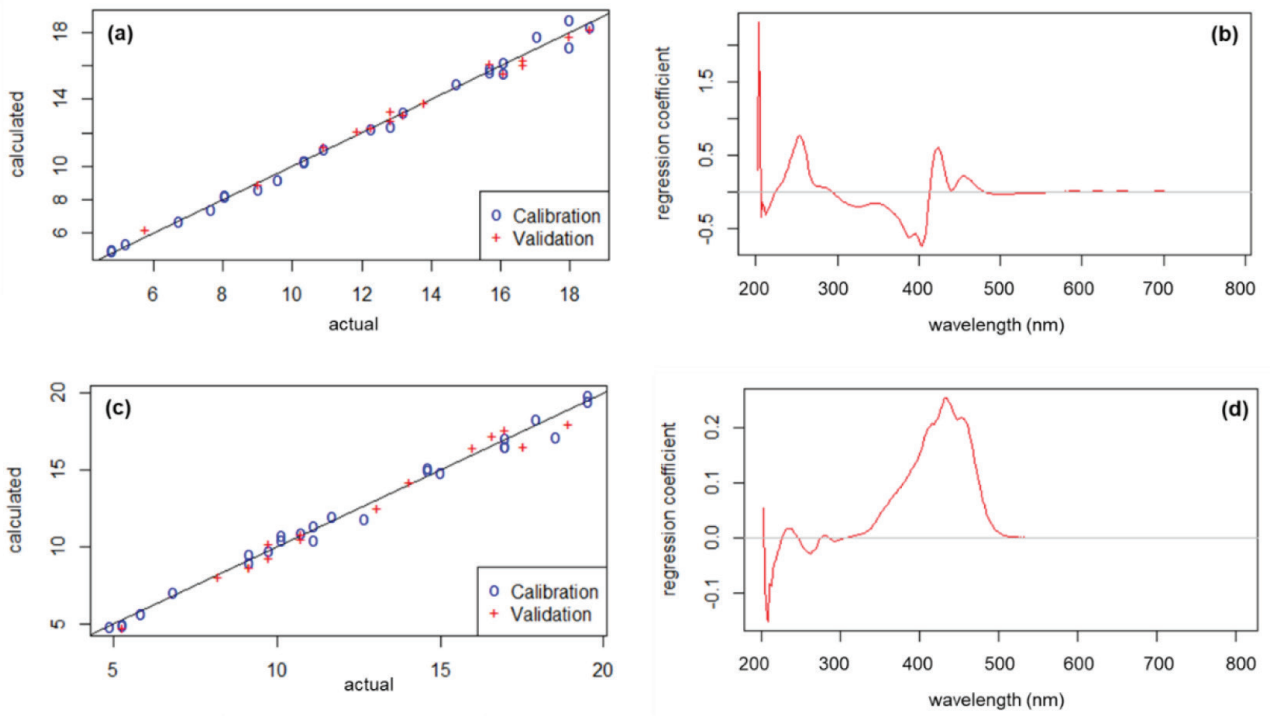

Figure 2. Multivariate calibration properties of selected model for genistein [prediction plot (a) and regression coefficient plot (b)] and curcumin [prediction plot (c) and regression coefficient plot (c)].

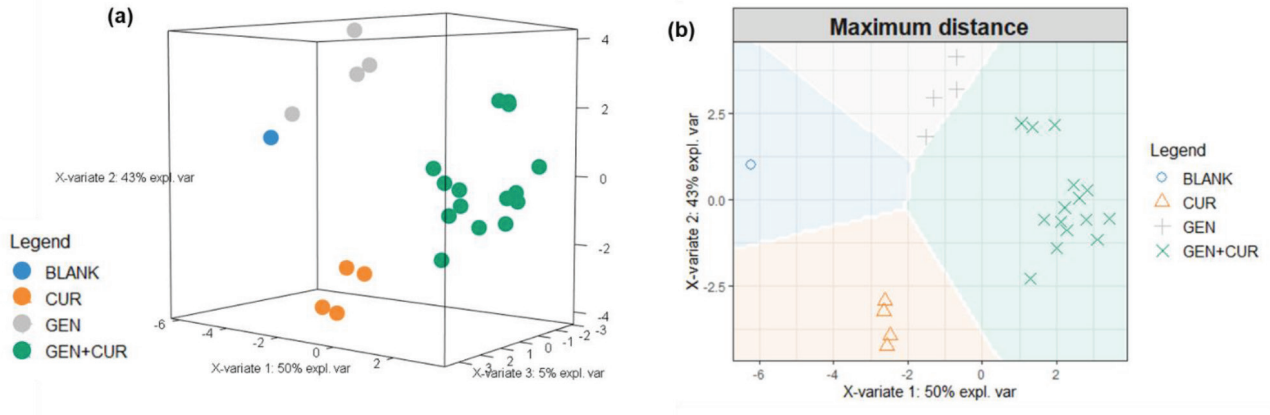

Figure 3. 3D visualization (a) and background prediction (b) of sPLS-DA performance generated using maximum prediction distance. BLANK = methanol; CUR = curcumin; GEN = genistein; GEN + CUR = binary mixture of GEN and CUR.

prediction distance. It means the maximum distance is applied to the predicted dummy variables and predicts the category of classes due to the largest dummy value (Rohart *et al*., 2017). It can be visually seen that the solvent, GEN, CUR, and binary mixture containing GEN and CUR were located in a separated region marked with different colors. However, this visualization was generated without any detailed parameters tuning. It is important to evaluate the sPLS-DA performance with a further model assessment.

The capability of the sPLS-DA model for discriminating between classes was evaluated using the AUROC which consisted of area under the curve (AUCuthe curve) and receiver operating characteristics (ROCpperating c). ROC represents probability and AUC represents the degree of separability of the model (Narkhede, 2018). Table 3 presents the results of the AUROC analysis of Component 1 and Component 2. As a common PLS model, the first two principal components contained the most information and variance compared to other components. With the higher AUC, a better model was obtained to distinguish between the true positive and true negative values. The solvent of BLANK and binary mixture of GEN and CUR were correctly discriminated compared to others. The chance of the sPLS-DA model to discriminate spectral patterns between CUR and others and GEN and others was 86.4% and 68.2%, respectively. However, the engagement of Component 2 in

Table 3. Results of the AUROC analysis on component 1 and component 2.

Outcome	Component 1		Component 2	
	AUC	*p*-value	AUC	*p*-value
Solvent versus others	1.000	5.625×10^{-3}	1.000	5.625×10^{-3}
CUR versus others	0.864	2.296×10^{-2}	1.000	1.766×10^{-3}
GEN versus others	0.682	2.555×10^{-1}	0.966	3.571×10^{-3}
GEN + CURcurcumin versus others	1.000	1.854×10^{-5}	1.000	1.854×10^{-5}

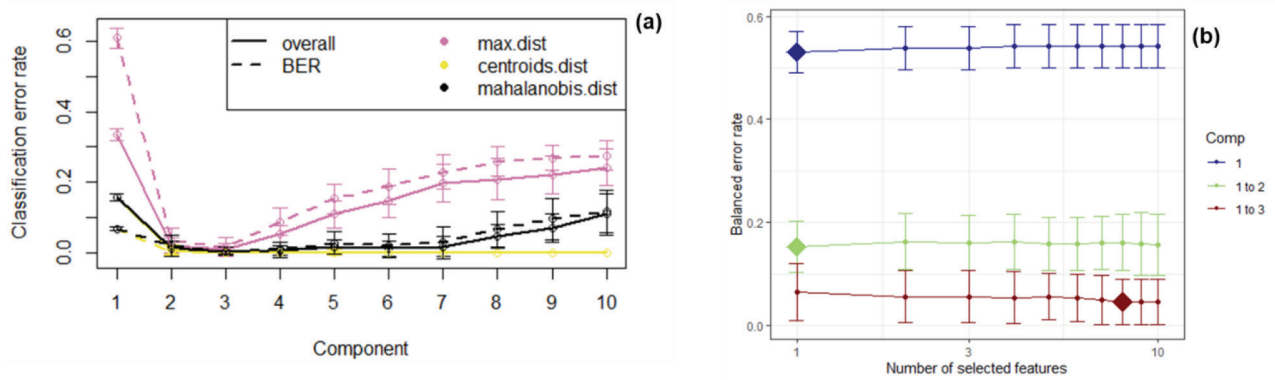

Figure 4. Classification error rate (a) and BER (b) plot from sPLS-DA tuning process.

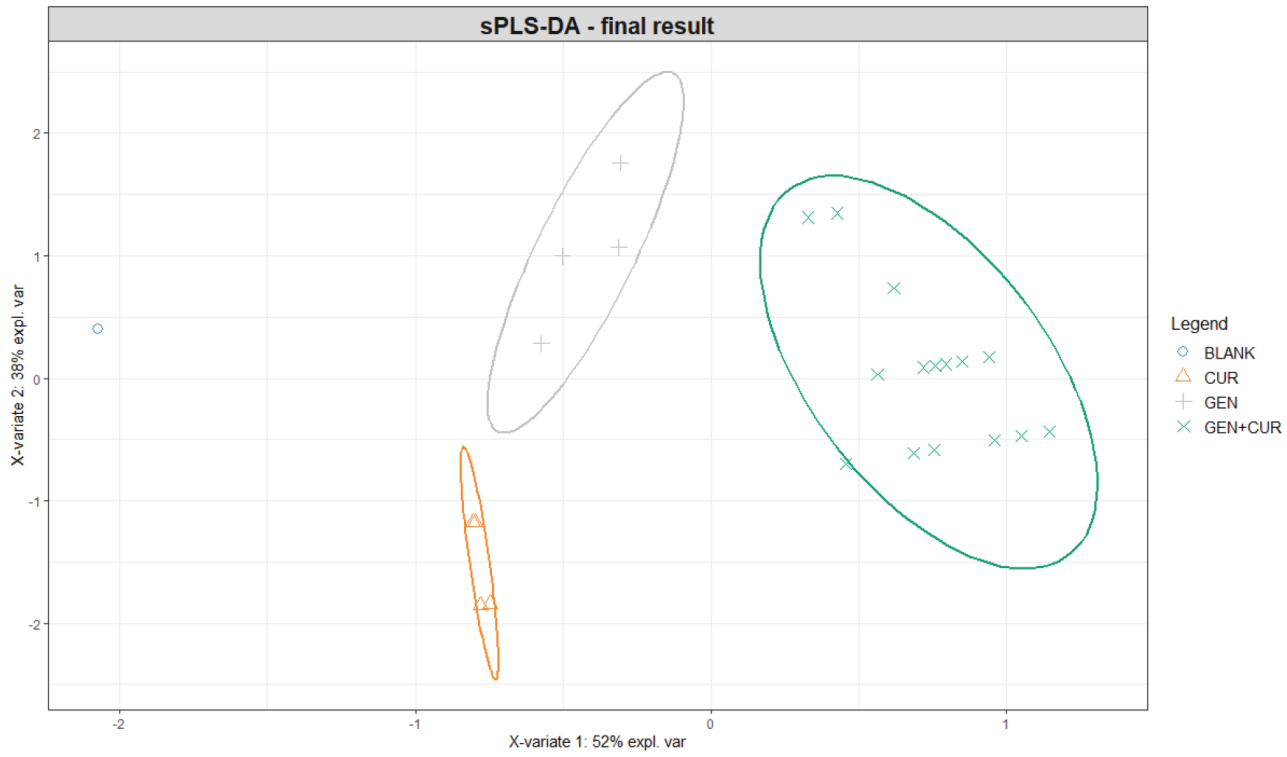

Figure 5. Final individual plot results of tuned sPLS-DA model. BLANK = methanol; CUR = curcumin; GEN = genistein; GEN + CUR = binary mixture of GEN and CUR.

generating the sPLS-DA model improved the chance of the model to distinguish GEN from other classes up to 96.6%.

Tuning parameters and numerical outputs of the sPLS-DA model were carried out in this study to implement the repetition and stratification of cross validation for model comparison. Three parameters should be chosen: the number of components, the number of variables "keepX" to select on each component, and the prediction distance. The cross-validation process was executed by applying 5-fold cross validation repeated 30 times. Figure 4 shows the classification error rate (a) and BER (b) plot from sPLS-

DA tuning process. Ten first components were stated in the list of initial "keepX" as variables. The number of components of three was selected since the classification error rate graph showed the lowest error rate towards all types of prediction distance. Balance error rate graph illustrates that the suggested list of "keepX" for three components was 1, 1, and 8.

Figure 5 shows the final individual plot after the sPLS-DA tuning model. All classes including BLANK, GEN, CUR, and GEN CUR mixture were explicitly separated. In addition, based on our findings, the parameters and numerical output tuning can be contextually applied to improve sPLS-DA model performance with the consideration of its error rate towards classification ability.

CONCLUSION

UV-Vis spectroscopy combined with multivariate calibrations was successfully generated for simultaneous determination of binary mixture containing GEN and CUR without any separation process. Multivariate calibration models for GEN and CUR were PCR of SG spectra and PCR of original spectra, respectively. The development of sPLS-DA as a statistical classifier method allowed the discrimination of GEN, CUR, and their mixture, as well as BLANK as a solvent. The parameters and numerical outputs tuning contributed not only to evaluating sPLS-DA model but also to enhancing its performance by selecting the minimum error rate for generating the final model.

This UV-Vis method and chemometrics techniques' combination proved to be rapid, simple, effective, and low cost compared to other methods with a separation process such as HPLC or HPTLC. However, it is quite interesting to develop spectroscopic or other analytical methods employed with chemometrics technique using natural product samples, for example, the combination of soybean and *Curcuma longa* L. extracts containing GEN and CUR.

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CONFLICT OF INTEREST

All the authors declared there is no conflict of interest.

AUTHORS' CONTRIBUTION

Florentinus Dika Octa Riswanto carried out the main laboratory works and initial publication writing. Abdul Rohman supervised the experimental works in the field of chemometrics. Suwidjiyo Pramono supervised the manuscript and data analysis. Sudibyo Martono provided the research funding, stated the conceptualization, and gave the final approval for publication.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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