ANTI-OXIDANT AND ANTI-CANCER. In this research, vitexin was used as a marker in the standardization of binahong leaf extract.

Pharmacological effects, such as anti-diabetes [19], anti-cancer, anti-oxidant, anti-virus, anti-inflammatory, anti-hypertension, etc [20]. It reported and obtained from binahong such as ursolic acid, ancordin, saponins and alkaloids [17]. Several active compounds were reported. The most common analytical method used was HPLC [21, 22]. Results of these study indicate that the determination of vitexin levels by the HPLC method has been well validated. However, this kind of method requires considerable expertise, spends a lot of time and requires equipment and costs. Therefore, it is necessary to develop a simple, efficient, and low-cost analytical method to determine the levels of vitexin in binahong extract.

The objective of this study was to develop a UV spectroscopy method in combination with multivariate analysis for determining vitexin in binahong (Anredera cordifolia (Ten.) Steenis) leaves extract.

METHODS: The partial least square (PLS) regression and the principal component regression (PCR) was performed in this study to evaluate several statistical performances such as coefficient of determination (R²), root mean square error of calibration (RMSEC), root mean square error of cross-validation (RMSECV), root mean square error of prediction (RMSEP) and relative error of prediction (REP). Cross-validation in this study was performed using leave one out technique.

RESULTS: The R² values of calibration data sets resulted from PLS and PCR method were 0.9675 and 0.9648, respectively. The low values of RMSEC and RMSECV both for PLS and PCR method indicated the minimum error of the calibration models. The R² values of validation data sets resulted from PLS and PCR method were 0.9778 and 0.9820, respectively. The low values of RMSEP both for PLS and PCR method indicated the minimum error of prediction generated from the calibration data sets. Multivariate calibration techniques were applied to determine the content of vitexin in binahong leaves extract. Predicted values from the multivariate calibration models were compared to the actual values determined from a validated HPLC method. It was found that PLS models resulted in the lowest REP values compared to the PCR models.

CONCLUSION: The chemometrics technique can be applied as an alternative method for determining vitexin levels in the ethanol solution of binahong leaves extract.

Keywords: Binahong, HPLC, UV Spectroscopy, Vitexin

INTRODUCTION

Binahong (Anredera cordifolia (Ten.) Steenis), one of 6000 types of plants in Indonesia, have been used for several health treatments [1]. Binahong has pharmacological effects such as anti-bacterial [2-5], anti-fungal [6], anti-aging [7], anti-diabetes [8], wound healing [9-11], anti-inflammatory [12], anti-oxidant [13], anti-cancer [14], anti- obesity [15] and anti-hyperuricemia [16]. Due to its pharmacological activities, binahong was potential to be developed as traditional medicine. Hence, it is important to determine the chemicals contained in binahong.

Binahong leaves contain terpenoids, steroids, glycosides, flavonoids, saponins and alkaloids [17]. Several active compounds were reported and obtained from binahong such as ursolic acid, ancondin, apigenin [17] and vitexin [13, 18]. Vitexin was reported due to its pharmacological effects, such as anti-diabetes [19], anti-cancer, anti-oxidant, anti-virus, anti-inflammatory, anti-hypertension, etc [20]. It can be stated that vitexin, one of the active compounds in binahong, can be potentially developed as an anti-diabetic, anti-inflammatory, anti-oxidant and anti-cancer. In this research, vitexin was used as a marker in the standardization of binahong leaf extract.

In previous studies, the determination of vitexin levels has been reported. The most common analytical method used was HPLC [21, 22]. Results of these study indicate that the determination of vitexin levels by the HPLC method has been well validated. However, this kind of method requires considerable expertise, spends a lot of time and requires equipment and costs. Therefore, it is necessary to develop a simple, efficient, and low-cost analytical method to analyze vitexin content in the binahong extract.

The use of spectroscopy, combined with the multivariate technique, was expected to overcome this problem. This study aims to determine the levels of vitexin in binahong (Anredera cordifolia (Ten.) Steenis) leaf extracts using spectrophotometry combined with multivariate techniques.

MATERIALS AND METHODS

Materials and Chemicals

The vitexin (Sigma-Aldrich, Missouri, USA), methanol HPLC grade (Merck Milipore, Germany) and orthophosphoric acid p. a. (Merck Milipore, Germany) was purchased from a local store in Yogyakarta, Indonesia. Redistilled water was obtained from the Analytical Instrument Laboratory, Faculty of Pharmacy, Universitas Sanata Dharma, Indonesia. Samples of binahong leaves were obtained from Wirobrajan district in Yogyakarta, Indonesia. The identification of Binahong leaves was confirmed as per the specimen authentication certificate no. 14.0.11/UM/FFA/BF/P/FT/2019 that was conducted by Biological Pharmacy Department, Faculty of Pharmacy, Universitas Gadjah Mada.

Instrumentation and Software

A set of UV-Vis Spectrophotometer (Shimadzu®, Kyoto, Japan) type UV 1800 equipped with quartz cuvette 1 cm (Hellma®, Jena, Germany), ultra-micro analytical balance (RADWAG®, Radom, Poland) series of UYA 2.3Y (max: 2.1 g, min 0.01 mg), a system of Shimadzu®, Kyoto, Japan) LC-2010HT No. C21255111004 LP with UV/Vis detector, (Retsch®, Haan, Germany) T460 ultrasonicator, membrane filter holder of Whatman® (Maidstone, United Kingdom) (capacity of 300 ml) Cat. No. 1960-004, organic solvent membrane filter of Whatman® (Maidstone, United Kingdom) (0.5 µm pore size, 47 mm diameter); inorganic solvent membrane filter of Whatman® (Maidstone, United Kingdom) (0.45 µm pore size, 47 mm diameter), Millipore® (Darmstadt, Germany) syringe filter (0.20 µm pore size, 25 mm diameter) and a set of Socorex® (Ecublens, Switzerland) micropipettes were used in this study.

Chemometrics Method

Extraction of Dry Leaves of Binahong

This procedure was developed from Yuliani et al. [23] and conducted as follow: 20 g dry leaves of binahong were weighed,
powdered and macerated using 200 ml ethanol for 90 min at 50 °C and shake at 200 rpm. The macerate was separated and concentrated into 25% of volume. This solution was labelled as the stock solution of ethanolic extract of binahong.

Preparation of calibration set and validation set solutions

Calibration and validation set solution was prepared by diluting a stock solution of ethanolic extract of binahong to achieve 26 compositions of samples as presented in table 1. Each composition was scanned using UV spectrophotometer at the range of 200 nm-400 nm. The absorbance for each composition was recorded with an interval of 2 nm. The obtained absorbance data for each wavelength were used for generating both calibration and validation models. The models were developed by plotting concentration of vitexin in each composition which was determined using HPLC as actual values vs the UV spectrophotometer predicted value.

Table 1: Calibration and validation data sets information for model selection and statistical analysis for determining the content of vitexin in ethanolic extract of binahong

| Items | Data sets | Calibration | Validation |
|-------|-----------|-------------|------------|
| Number of mixture standards | 16 | 10 |
| Vitexin concentration in ethanolic extract of binahong (µg/ml) | 14.12 | 13.11 |
| Mean | 6.38-26.72 | 5.36-25.42 |
| Range | PCR | PCR |
| Multivariate calibration models | PLS | PLS |
| Evaluated parameters for model selection | R² | R² |
| | RMSEC | RMSEP |
| | RMSECV* |

Note: *cross-validation was performed using leave one out (LOO) technique

Spectroscopic analysis and multivariate calibrations

The absorbance values of every single wavelength point achieved from the calibration and validation data sets were statistically analyzed using the R studio software. A statistical package of pls was employed to perform chemometrics data processing. This package has been downloaded and installed by using the function of install. Packages ("pls"). After successfully installed, this package was loaded by using the function of library (pls) before further statistical analysis. A couple of multivariate calibration models, namely PLS and PCR, was generated for ethanolic extract of binahong. The built model multivariate calibration models were statistically evaluated by assessing several performances such as coefficient of determination (R²), root mean square error of calibration (RMSEC), root mean square error of cross-validation (RMSECV), and root mean square error of prediction (RMSEP). Further, multivariate calibration model for each compound which resulted in a value of R² near one and lower RMSEC, RMSECV, and RMSEP were chosen for content determination [24].

Preparations of samples

Samples of diluted ethanolic extract solution of binahong were taken 1 ml. All the diluted and replicated sample solutions were sonicated for 15 min and filtered using Milipore syringe filter before injection. These prepared samples were analyzed both using spectroscopy method for generating the predictive models of multivariate calibration and HPLC method for achieving the actual values for each compound containing in the samples.

HPLC method

Chromatographic conditions

The system of Shimadzu LC-2010 CHT with LabSolution software and UV-ViE detector was developed. This chromatographic system was developed from Mulia et al [25] with the column type, flow rate, pH, and stop time modification. A C18 column of Luna type Phenomenex (250 mm x 4.6 mm, 5 µm) was used. An isocratic elution system was developed. The composition of the mobile phase was methanol-acetonitrile-water (adjusted until pH 3 by orthophosphoric acid) (20:20:60), and mobile phase flow rate was 0.8 ml/min. Chromatographic separation was performed at ambient temperature the injection volume of 10 µl, and stop time setting at 25 min. Quantitation was set with UV detection at 319 nm.

Preparation of vitexin standard solutions

Accurate weight of 0.1 mg of vitexin was transferred into 5 ml volumetric flask. The weighted standard in the volumetric flask was diluted in methanol into the volume. These solutions were labelled as vitexin stock solution.

Standard solutions for UV spectral scanning were prepared from the stock solution. The stock solution was transferred into three separated 5 ml volumetric flasks, respectively, followed by the methanol dilution into the volume.

System suitability parameters

System suitability parameters were analyzed to check the system performance consistency. For system suitability parameters, six times repetition of one concentration of calibration solution vitexin was injected, and column performances like area, retention time, tailing factor, and a number of theoretical plates were observed. Values of the percentage of relative standard deviation (% RSD) for area and retention time parameters were found within the acceptance criteria of system performance. Tailing factor and number of theoretical plates were found for qualitative study based on USP [26].

Analytical method validation

The analytical method for determining the content of vitexin was validated for selectivity, linearity, range, precision, accuracy, detection limit, and quantitation limit, according to the ICH guidelines [27] and AOAC [28].

RESULTS

Validation method and analysis of vitexin in ethanolic extract of binahong using HPLC

The representative chromatogram profile of the sample and vitexin were presented in fig. 1 and 2. The HPLC performance was shown in table 2.

Chemometrics application

The spectra of calibration and validation set solutions can be seen in fig. 3 and 4, while table 3 and 4 presented the performance of PLS and PCR in the calibration and validation solutions. The results of calibration and validation with PLS and PCR can be seen in fig. 5 and 6.
Fig. 1: The representative chromatogram profile of the sample (binahong)

Fig. 2: The representative chromatogram profile of the vitexin (10 ppm)

Table 2: The performance of the validation method and analysis of vitexin in ethanolic extract of binahong using HPLC

| Parameters                                      | Value   | Acceptance criteria |
|------------------------------------------------|---------|---------------------|
| System suitability test                         |         |                     |
| % RSD Tr (min)                                  | 0.38    | % RSD<2%            |
| % RSD Area                                      | 0.12    | T<2.0               |
| Tailing factor (Tf)                             | 1.247   | N>2000              |
| Number theoretical plate (N)                    | 7488    |                     |
| Selectivity                                     |         |                     |
| Resolution (Rs) sample                         | Rs = 2.045 | Resolution>1.5 |
| Linearity                                       |         |                     |
| Coefficient correlation (r)                     | 0.9996  | 0.999               |
| Coefficient determination (R)                   | 0.9992  |                     |
| Slope (b)                                       | 20349   | -                   |
| Intercept (a)                                   | -28532  | -                   |
| Range                                           | 2.11 ppm–31.61 ppm | -             |
| Limit of detection                              | 1.10 ppm | -                   |
| Limit of quantitation                           | 3.34 ppm | -                   |
| *Accuracy (Recovery)                            |         |                     |
| (intra-day)                                     | 97.85%–104.42% | 95%–105%          |
| (inter-day)                                     | 101.45%–103.40% |                     |
| *Precision (RSD)                                |         |                     |
| (intra-day)                                     | 1.63%–3.04% | <3.7%              |
| (inter-day)                                     | 1.35%–3.33% |                     |
| *Assay content vitexin in ethanolic extract of  |         |                     |
| binahong                                        | 0.11±0.01 % w/w with RSD = 1.32% | RSD<3.7%         |

Note: * Accuracy and Precision were determined at 3 concentration addition levels of vitexin, 3 replicates and 3 consecutive days for inter-day. +Assay content was determined by 3 replicates of ethanolic extract of binahong.
Fig. 3: The spectra of calibration set solutions

Fig. 4: The spectra of validation set solutions

Table 3: The performance of partial least squares (PLS) and principal component regression (PCR) for predicting the content of vitexin in calibration set solutions

| Compounds | Multivariate calibrations | Calibration Number of components | $R^2$ | RMSEC | RMSECV |
|-----------|--------------------------|---------------------------------|-------|-------|--------|
| Vitexin   | PLS                      | 11                              | 0.9675| 0.0738| 1.204  |
| Vitexin   | PCR                      | 14                              | 0.9648| 0.0585| 1.253  |
Table 4: The performance of partial least squares (PLS) and principal component regression (PCR) for predicting the content of vitexin in validation set solutions

| Compounds | Multivariate calibrations | Validation | \# of components | $R^2$  | RMSEP  |
|-----------|--------------------------|------------|------------------|-------|--------|
| Vitexin   | PLS                      | 11         | 0.9778           | 1.2437|
| Vitexin   | PCR                      | 14         | 0.9820           | 1.1669|

Fig. 5: Observation data plot (actual) vs predicted data (calculated) with PLS

Fig. 6: Observation data plot (actual) vs predicted data (calculated) with PCR

Table 5: The assay of vitexin at the ethanolic extract of binahong leaves result

| Compounds | Method | \# of solution test | REP  |
|-----------|--------|---------------------|------|
|           |        | 1                   | 2    | 3    |       |
| Vitexin   | PLS    | 8.192513            | 16.950112 | 12.530707 | 5.603%|
| Vitexin   | PCR    | 7.95943             | 17.01199 | 12.12951 | 6.917%|
| Vitexin   | Actual | 9.29                | 16.32  | 12.57 | -     |
The assay of vitexin

Result of the assay of vitexin at the ethanolic extract of binahong leaves can be seen in table 5.

DISCUSSION

The HPLC method was chosen as a reference method, according to the previous study [25]. This reference method was modified and validated due to the different parameters of the instrument compared to the previous study. The representative chromatogram profile of the sample was presented in fig. 1. This study was validated according to ICH (selectivity, linearity, range, the limit of detection, the limit of quantitation, accuracy and precision) [27]. Parameters of system suitability test and validation study were presented in table 2.

The actual data of vitexin levels in binahong leaves extract should be determined to conduct a multivariate analysis. The actual data was obtained from the HPLC analysis. The results of the validation method for determining levels of vitexin by HPLC are presented in table 2. The system suitability test and all validation parameters have been met with the requirement with high sensitivity, since the LoD and LoQ were 1.10 ppm and 3.34 ppm, respectively. This method gave valid, simple and fast method to determine vitexin in binahong if it was compared to Dwitiyanti et al. [29] they reported using gradient elution to determine vitexin compound in binahong, but in this study, isocratic elution was developed to determine vitexin compound. Mulia et al. [25] also reported HPLC method to determine vitexin in binahong extract, but they use no ethanol as a solvent to extract the isocratic elution to determine vitexin compound. Mulia et al. [25] and Dwitiyanti et al. [29] also reported no validation data in their research, so this method can be made an alternative method to standardize binahong extract.

The output of UV spectral data was exported to Excel (Microsoft) and converted into. csv formatted files for further chemometrics data processing. Statistical analysis and multivariate calibrations were performed using R Studio software version 1.1.456 with pk packages. This software can be freely downloaded from https://rstudio.com/products/rstudio/download/.

The results of R² both for PLS and PCR were 0.9785 and 0.9802, respectively. These coefficient correlations indicated that the predicted value (model) and the observed value (HPLC) resulted in a very good correlation. RMSEC values resulted from the PLS and PCR models were 0.0738 and 0.0585, respectively, while RMSECV obtained from LOO cross-validation for PLS and PCR models were 1.204 and 1.253, respectively. Less values both for RMSEC and RMSECV indicated that minimum errors in calibration models.

The results of calibration and validation with PLS and PCR can be seen in fig. 5 and 6. Table 4 presented the performance of PLS and PCR in the validation solution that resulted in R² of 0.9778 and 0.9820, respectively. These results indicated that the predicted value and observed values showed a good correlation. RMSEP values given by the PLS and PCR models were 1.02437 and 1.02437, respectively. Hence, it can be assumed that the generated model resulted in minimum errors of prediction.

Spectral data from the measurement results of the samples were collected and processed statistically to achieve the prediction results of vitexin levels. The prediction results were then calculated by the REP value against the results of the determination of levels by HPLC, as shown in table 5.

Based on the results of the determination of the levels of vitexin in the ethanol extract solution of binahong leaves (see table 5), by experimenting with 3 sets of test solutions, the results using the PLS model showed values of 8.192513, 16.950112 and 12.530707, respectively. When compared with the actual value (HPLC), an error value (REP) of 5.603% is obtained, which means very good. Likewise, the results using PCR showed successive values of 7.95943, 17.01199 and 12.12951. When compared with the actual value (HPLC), an error value (REP) of 6.917% is slightly greater, but it can still be said to be very good.

CONCLUSION

The UV spectroscopy combined with chemometrics technique can be developed as an alternative method for determination of vitexin levels in the ethanol solution of binahong leaves extract.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

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