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ISSN 1755-6783

Annals of Tropical Medicine and Public Health

www.atmph.org

Official Publication of Africa Health Research Organization

Volume 23 | Issue 3 | February 2020

Wolters Kluwer Health | Medknow

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Application of near infra red (NIR) spectroscopy and chemometrics for determination antioxidant activity of plant leaves extracts

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Abstract.
Context: Near Infra red (NIR) spectroscopy combined with chemometrics has been developed for simple analysis of antioxidant activity in the medicinal plant extract. **Aims**: The aim of this research was to study whether NIR and chemometric methods could be used to determine the antioxidant activity. **Settings and Design**: A model for determining antioxidant activity of plant extracts was formed using chemometric and the model was applied on real samples. **Methods and Material**: Medicinal plant leaves were extracted and its spectral data were correlated with its antioxidant activity using chemometric. The chemometric method used for calibration analysis were Partial Last Square (PLS), Principal Component Regression (PCR) and Support Vector Machines Regression (SVMR), and the methods used for classification analysis were Linear Discriminant Analysis (LDA), Soft Independent Modelling of Class Analogies (SIMCA), and Support Vector Machines Classification (SVMC).**Statistical analysis used**: Paired–sample t test was used in this study. **Results**: In this study, SVMC that showed best classification with accuracy was 100 % and SVMR that showed best calibration with R2 and RMSEC value was 0.9 811 205 and 4.4 940 028, respectively. SVMC and SVMR models were further used to predict unknown antioxidant activity in commercial and simulation samples. Using these models, the significance of antioxidant activity that has been measured by NIR and UV–Vis spectrophotometry was evaluated with paired samples ttest and gave no significant difference. **Conclusions**: This study showed that NIR spectroscopy coupled with chemometric could be used for determining antioxidant activity of several plant extracts.

Keywords: Rapid antioxidant activity analysis, multivariate analysis, chemometrics model

How to cite this article: Wulandari et al. (2020): Application of near infrared (NIR) spectroscopy and chemometrics for determination antioxidant activity of plant leaves extracts, Ann Trop & Public Health; S464 Vol. 23 Issue 3(A): 68–77.
DOI : http://doi.org/10.36295/ASRO.2020.23317

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Introduction

Leaves are part of plants that have an important role and have the main function of carrying out photosynthesis to produce food[1]. In–plant cells that experience photosynthesis, many phenolic compounds are found[2]. It has been widely known that phenolic compounds are a source of natural antioxidants. Antioxidants are compounds that can delay or prevent the occurrence of free radical oxidation reactions that play a role in the pathology of various degenerative diseases[3].

The analytical method that can be used to identify antioxidant activity is by using Diphenylpicrylhydrazyl (DPPH) method, radical nitrogen monoxide inhibition activity method, 2,2'–azino–bis(3–ethylbenzothiazoline–6–sulphonic acid) (ABTS) method, Ferric Reducing Ability of Plasma (FRAP) method, oxygen radical absorption capacity method (ORAC), reducing strength method, xanthine oxidase method, radical inhibitory activity method hydroxyl, and lipid method microsomal peroxidation[4]. However, these methods require a lot of energy and time, so it is necessary to develop analytical techniques that are faster, more effective and trustworthy.

Near Infra red Spectroscopy (NIR) is one of the effective methods because it is a non–destructive analysis technique, can analyze at high speed, does not cause pollution and does not require chemicals[5]. However, the NIR spectrum band is very complicated and overlapping. Therefore a chemometric technique such as multivariate analysis is needed to interpret it[6]. Chemometric benefits are the ability to know spectrum information from the infrared spectrum and use spectrum information for qualitative and quantitative applications[7].This study aims to determine whether the NIR spectroscopy and chemometric can be used to determine the antioxidant activity seen from the value of Inhibition. Concentration 50 % (IC_{50}), determine the antioxidant activity in real samples and identify the significance and results of IC_{50} count with infrared spectrum compared to the comparison method.

Materials and Methods

Material and reagent

The tools used in this study were UV–Vis (Hitachi U–1 800) spectrophotometer, near–infrared spectrophotometer (Brimrose Luminar 3 070), Brimrose software, The Unscrambler × 10.2 (Camo) software, ultrasonicator (Elmasonic), Rotavour, ovens, digital analytic scales, blenders, erlenmeyer, funnels, porcelain dishes, extract spoons, filter paper, and glassware.

The sample was chosen based on library data of antioxidant activities that were owned. The sampling technique uses was purposive sampling taking into varying the capability of antioxidant activity. Plants leaves were collected from *Unit Pelaksana Teknis Materia Medika* (Materia Medika Technical Implementation Unit), Batu City, Malang (Table 1). The real sample from commercial products were stimuno capsules® (Dexa Medica), bay leaf capsules® (Indo Main Herbs),

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soursop leaf capsules® (Indo Main Herbs). The reagents used were methanol pa (Merck), aerosil (pharmaceutical grade), DPPH (Sigma–Aldrich), ascorbic acid (pharmaceutical grade), filter paper, aluminum foil.

Table 1. Plant leaves used

No	Code	Name of Plant
1	E	*Kaempferia rotunda* L.
2	F	*Mangifera indica* L.
3	I	*Phaseolus vulgaris* L.
4	Q	*Carica papaya* L.
5	K	*Morinda citrifolia* L.
6	G	*Euphorbiae hirtae* L.
7	J	*Sauropus androgynus* M.
8	N	*Piper betle* L.
9	O	*Annona muricata* L.
10	P	*Pandanus amaryllifolius* R.
11	D	*Artocarpus camansi* B.
12	L	*Momordica charantia* L.
13	C	*Syzygium cumini* L.
14	H	*Ruellia tuberose* L.
15	M	*Piper crocatum* Ruiz & Pav.
16	R	*Coffea canephora* L. (Young)
17	S	*Coffea canephora* L. (Old)
18	A	*Coffea arabica* L. (Young)
19	B	*Coffea arabica* L. (Old)
20	T	*Azadirachta indica* A.
21	V	*Mimosa pudica* L.
22	U	*Ocymum basilicum* L.

Extraction

A total of 80 g of dried leaf powder was ultrasonified in 800 mL of 98 % methanol for 1 h. The extraction results are then left to stand for 24 h. The filtrate obtained was concentrated with a rotary evaporator so that a thick extract was obtained. The thick extract was then dried with aerosil. Furthermore, the dried extract was crushed until smooth and sifted with a B–60 sieve.

Spectra aquisition of NIR spectroscopy

The NIR instrument was turned on and warmed for thirty minutes. Open the Brimrose software and then the sample was placed on the sample plate evenly and compactly. Sample was scanned five times and three shootings were performed at each scan at 850 nm to 2 000 nm. The step is repeated for each sample. The data that has been obtained is processed with the software Unscrambler × 10.2.

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Determination of antioxidant activity

Preparation of the test solution with five concentrations varies. Measurement of antioxidant activity carried out by means of 1.2 mL of 0.1 mm DPPH was added with 0.3 mL of each test solution. The mixture is shaken until homogeneous and then incubated in a dark place. Furthermore, its absorption is measured at a wavelength of 515.5 nm. The IC_{50} value is calculated based on the DPPH radical reduction percentage of each sample solution concentration which follows the equation(1):

$$\%\ \text{inhibition} = \frac{\text{Blank Absorbance} - \text{Test Solution Absorbance}}{\text{Blank Absorbance}} \times 100\ \% \qquad (1)$$

After obtaining the inhibition percentage of each concentration, followed by the calculation by linear regression using the equation(2):

$$y = a + bx \qquad (2)$$

Antioxidant activity was expressed by 50 % inhibition concentration or IC_{50}, which is the concentration of samples which can reduce DPPH radicals by fifty concentration units in $\mu g\ mL^{-1}$.

Determination calibration and classification models

The calibration model for quantitative analysis in this study was formed by correlated the spectras data with IC_{50} values then analyzed using PLS,PCR and SVMR. The classification model was formed by correlated the spectras data with categorizes (has antioxidant activity and no antioxidant activity) then analyzed using LDA, SIMCA, and SVMC. Each of the best models that have been formed was validated using two cross validation techniques, the first technique was Leave One Out Cross Validation (LOOCV) by processing the training data set in The Unscrambler × 10.2 software and the second technique was cross validation of 2–Fold–Cross Validation that used an independent samples called test set. The test set extract must be different extract from the extract used in the training set.

Application in real extract samples

After validation of the models then applied to the determination of IC_{50} values of real samples and simulation samples. Real samples and simulation samples were scanned using NIR spectroscopy and determined the IC_{50} value from the model obtained. The IC_{50} value obtained is then compared with the IC_{50} value obtained from the comparison method. IC_{50} values obtained from the two methods were then tested by ttest two paired samples to determine whether there were differences in IC_{50} values given by both.

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Result

Antioxidant activity of each plants were determined with UV–Vis spectrophotometry method, and the results were presented in Table 2. Ascorbic acid was used as the standard. The antioxidant activity of the plants tested was measured in IC_{50} and the value was ranged from 6.188 µg mL^{-1} to 911.9 µg mL^{-1}.

Spectral data was then obtained from NIR analysis, then, with the IC_{50} data, calibration and classification models were formed with chemometric. The results were presented in Table 3 and Table 4. Classification model's Root Mean Square Error (RMSE) and R–Square value were varies. The accuracy of the classification model were ranged from 32.90 % to 100 %, with SVMC model achieving the highest accuracy of 100 %.

Table 2. IC_{50} of sample (plant extract) and standart (ascorbic acid)

No	Kode	IC_{50}(µg mL^{-1})	% RSD (n = 3)
1	Ascorbic acid	3.396	1.39
2	A	18.63	0.60
3	B	14.17	1.70
4	C	41.25	0.23
5	D	32.88	1.14
6	E	113.2	0.75
7	F	6.188	1.05
8	G	14.99	0.42
9	H	28.15	1.52
10	I	528.1	0.89
11	J	329.2	0.43
12	K	937.7	2.03
13	L	489.648	0.14
14	M	388.755	1.83
15	N	911.892	0.74
16	O	285.575	1.92
17	P	386.315	0.90
18	Q	614.024	1.28
19	R	11.046	1.09
20	S	12.310	0.24

Table 3. Result of calibration models

Calibration Models	Root Mean Square Error		R-Square	
	Calibration	Validation	Calibration	Validation
PLS	8.1 782 084	8.4 771 109	0.9 311 977	0.9 270 415
SVMR	4.4 940 028	5.2 207 675	0.9 811 205	0.9 741 873
PCR	9.0 157 061	9.3 416 862	0.9 163 846	0.9 113 821

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Table 4. Result of classification models

Classification Models	% Accuracy
LDA	74.03 %
SVMC	100 %
SIMCA	32.90 %

Discussion

In this research antioxidant activity evaluated using DPPH which acts as a free radical. Free radicals are then suppressed by antioxidants from the test material, where DPPH will be captured by antioxidants by donating hydrogen atoms from antioxidants to form reduced DPPH–H. This reaction causes a color change that can be measured by a UV–Vis spectrophotometer at a wavelength of 515 nm to 520 nm in an organic solvent (methanol or ethanol)[8]. Addition of antioxidants with various concentrations will remove the purple color and gradually turn yellow according to the concentration of antioxidants. Percent inhibition will increase with increasing concentration of extract[9]. The concentrations of extracts and comparators reacted are plotted with percent reduction. Furthermore IC_{50} values are obtained. IC_{50} is an effective concentration that can reduce DPPH free radicals by 50 %.

Based on the results of the determination of antioxidant activity in plant leaf extract by UV–Vis spectrophotometry method, showed the greatest antioxidant activity, namely in mango leaf extract with IC_{50} value of 6.19 $\mu g\ mL^{-1} \pm 1.05\ \mu g\ mL^{-1}$. The smallest antioxidant activity is Noni leaf extract with IC_{50} value of 937.7 $\mu g\ mL^{-1} \pm 2.03\ \mu g\ mL^{-1}$. The results of the determination of antioxidant activity of plant extracts can be seen in Table 2.

Infrared spectrophotometry is an analysis technique based on the vibrations of atoms in molecules. The advantages of infrared spectrophotometry is non–destructive, need small amout of samples, and almost all forms of samples can be investigated[10]. In addition, infrared spectrophotometric techniques require almost no chemical solvents so they are more environmentally friendly[11].

Spectral data generated from the determination of NIR data is used to form the chemometric classification and calibration model. The formation of the classification and calibration model in this study was formed from 16 training sets and four test sets. All absorbance data at wavelengths of 850 nm to 2 000 nm have been obtained. The parameters considered in selecting the best model in the calibration model are based on the value of R2 and the value of RMSEC. The value of R2 is the correlation value where the best model selection is if the correlation value obtained is greater and the RMSEC value is the lowest[12]. While the parameter classification model that is considered in selecting the best model is based on the value of accuracy. Comparison of the value of R2 and RMSEC value

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of each calibration model can be seen in Table 3 and the comparison of the values of each classification model can be seen in Table 4.

Based on the data obtained, SVMR was chosen as the best calibration model because it has the highest R2 value and has the lowest RMSEC. SVMC was chosen as the best classification model because it has a 100 % accuracy value which indicates that none of the samples were wrong in its categorization.

The truth of the calibration model and classification that is formed is then tested with cross validation. Cross validation techniques are used to predict or estimate how accurate the prediction model is made to be implemented[13]. For the validation process of the selected method, a validation process is performed with Leave One Out Cross Validation and two Fold Cross Validation. LOOCV is done by removing a set of data from the training set data where the remaining data is used to form the model and the sample that has been issued is predicted by the model. The value obtained in this study is R2 of 0.9 802 536, RMSE of 4.4 775 705 and accuracy of 100 %. Validation method two–Fold Cross Validation uses independent samples (using new samples outside the training set sample). In this study using four samples as a test set. The value obtained in this study is R2 of 0.9 884 and accuracy of 100 %.

The value of R2 in this validation method has a good ability in predicting concentration from the sample because the R2 value is formed above 0.91[14]. The results of validation indicate that the accuracy value is 100 % which means that the model can classify the training sample set correctly.

The SVMC and SVMR models that have been formed in the software Unscrambler × 10.2 are applied in the analysis of real samples to determine antioxidant activity using the NIR spectroscopic method. The results of the determination of antioxidant activity using two methods (Table 5) then calculated the significance value and the calculated t value using t test analysis of two samples in pairs. Statistical analysis with two paired samples t test of the data can be concluded that there were no significant differences in the level of real samples determined by the two methods ($p > 0.01$).

Table 5. IC_{50} Value of real sample and simulation sample

Name of Sample	IC_{50} (μg mL^{-1}) ± % RSD (n = 3)	
	NIR Spectroscopy (SVMR model)	UV-Vis Spectroscopy
Commercial product–1	125.2 ± 1.33	122.3 ± 0.04
Commercial product–2	252.3 ± 1.22	252.4 ± 0.25
Commercial product–3	301.1 ± 0.15	313.3 ± 0.04
Simulation extract–T	51.48 ± 0.95	70.64 ± 0.57
Simulation extract–U	136.8 ± 0.43	89.79 ± 0.62
Simulation extract–V	64.57 ± 2.02	34.62 ± 0.12

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Conclusion

This study showed that NIR spectroscopy coupled with chemometric could be used for determining antioxidant activity of several plant extracts.

Acknowledgement

The authors are thankful to the University of Jember for the research support.

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