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FTIR spectra-based fingerprinting and chemometrics for rapid investigation of antioxidant activities of medicinal plants

Ruslin RUSLIN [1], Yamin YAMIN [1], Irnawati IRNAWATI [1], Anjar WİNDARSİH [2], Abdul ROHMAN [3,4*]

1 Faculty of Pharmacy, Universitas Halu Oleo, Kendari 93232, Indonesia.
2 Research Center for Food Technology and Processing (PRTPP), National Research and Innovation Agency (BRIN), Yogyakarta, Indonesia.
3 Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, 55281, Indonesia.
4 Center of Excellence Institute for Halal Industry & Systems, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia.

* Corresponding Author. E-mail: abdul_kimfar@ugm.ac.id (A.R.); Tel. +62-878-3844 52 16.

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ABSTRACT: Various plants have been used as herbal medicines by the community since they contain a lot of phytochemicals having the beneficial effect to human health including flavonoids and phenolic compounds. The objective of this study was to evaluate the antioxidant activities, determine the levels of total phenolics and flavonoids contents in selected medicinal plant extracts and fractions. The plants were extracted with methanol, then fractionated with *n*-hexane and ethyl acetate to get extracts and its fractions. The extracts were subjected to FTIR spectral measurements antioxidant activities evaluation as well as total phenolics and total flavonoids contents. Furthemore, FTIR spectra assisted by chemometrics were used to predict these antioxidant activities. Ethyl acetate fraction of medicinal plants provided the strongest antioxidant activities with high levels of total phenolic and flavonoids. Partial least square regression (PLSR) using variable of absorbance values at 3600 – 650 cm^{-1} exhibited good correlation between actual values of antioxidant activities (IC_{50}) and predicted values with R^2 values of 0.9974 and 0.9983 in calibration and validation models, respectively. Furthermore, the low values of RMSEC (0.706) and RMSEP indicate that the developed models were precise enough to predict the antioxidant activities. The combination of FTIR spectroscopy and PLSR offered the accurate and precise models for the prediction of antioxidant activities of studied extracts and fractions of studied plants.

KEYWORDS: Chemometrics; DPPH antiradical assay; FTIR spectroscopy; herbal medicine; multivariate data analysis.

1. INTRODUCTION

The use of plants as raw materials in medicine has been known for a long time due to the safety issues of chemical drugs [1]. Various plants have been reported to be used as herbal medicine in folk communities such as (*Vernonia amygdalina* Del.) leaves [2], (*Chromolaena odorata* L.) leaves [3], (*Ipomea batatas* L.) leaves [4], (*Diplazium esculentum*) leaves [5] and (*Manihot esculenta*) [6] leaves. The community uses these medicinal plants as a source of antioxidants, essential in inhibiting the treatment of infectious diseases, diabetes, inflammation, hypertension, and cancer [7,8]. The medicinal plants contain many phytochemicals such as flavonoids, phenolics, terpenoids, alkaloids, tannins, xanthones, having the beneficial effects to human health, especially to prevent the degenerative diseases caused by oxidative stress, an imbalance between free radicals and endogenous antioxidants [9]. The phenolics and flavonoids compounds are believed as the most responsible phytochemicals for counteracting free radicals, therefore, plants with high contents of phenolics and flavonoids are considered as natural antioxidants [10].

Antioxidants are species or compounds, both natural and synthetic, that can significantly delay or prevent the oxidation of substrates at low concentrations compared to the oxidized substrates [11]. Due to

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the safety issues and side effects of using synthetic antioxidants, the exploration of natural sources of antioxidants, especially from plants, has continued to be developed in recent decades [12]. Various methods have been developed to evaluate antioxidant capacity of natural products *in vitro*, including those based on electron/hydrogen transfer, such as the 2,2-diphenyl-1-picrylhydrazyl method (DPPH assay) and a method of measuring antioxidant capacity known as the 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) or ABTS method, electron transfer–based assays, including ferry reduction antioxidant power (FRAP) and cupric ion reduction antioxidant power (CUPRAC) [13,14]. The DPPH radical scavenging method is the most popular used to measure the antioxidant activity of plant extracts [15].

Several studies have shown that there is a correlation between the results obtained during the evaluation of antioxidant activities in vitro with some phytochemicals such as phenolic compounds and flavonoids [16,17] and with the specific responses obtained during instrumental analysis. Measuring antioxidant activity using physicochemical tests (DPPH, ABTS, FRAP) typically involve the high amount reagents and solvents which are pollutant to the environment, therefore, some alternative methods have been developed, including using variable of Fourier transform infrared (FTIR) spectra. With the development of chemometrics or multivariate data analysis, some chemists try to correlate the antioxidant activity of samples with certain spectroscopic responses (absorbances) in order to make the classification and differentiation of plant materials [18,19]. Several studies have been successfully correlating the antioxidant activity using spectroscopic methods. Kusumadewi et al. [20] have applied FTIR spectra and chemometrics to classify *Curcuma xanthorrhiza* from Indonesia based on the antioxidant activities. Rafi et al. [21] identified and discriminated four Curcuma species using UV-Vis spectra combined with pattern recognition chemometrics of principal component analysis (PCA). The combination of chemometrics and NMR spectra has succeeded in characterizing mono-floral honey, specifically for New Zealand and Australia [22]. From various reference searches, research reports on the application of FTIR to classify medicinal plants from Southeast Sulawesi have yet to be found. Therefore, the purpose of this study was to classify several medicinal plants of Southeast Sulawesi based on FTIR spectra combined with PCA chemometrics and cluster analysis (CA) and to predict DPPH radical scavenging activity based on FTIR spectrum developed with multivariate calibration of partial least square calibration (PLS).

2. RESULT AND DISCUSSION

The antioxidant activities of several medicinal plants from Southeast Sulawesi, Indonesia as determined using DPPH, ABTS and FRAP assays exhibited very strong capacity to scavenge free radicals, both extracts and fractions, as shown in Table 1. The contents of total phenolics (TPC) and total flavonoid (TFC) in ethyl acetate fractions of the studied medicinal plants were generally higher than those in extracts and other fractions. The ethyl acetate fraction also showed the strongest antioxidant capacity compared to extracts of methanol, *n*-hexane fraction, and water fraction as indicated by the low IC_{50} values. The lower IC_{50} values, the stronger of antioxidant activities. This finding was in line with several previous studies, including the ethyl acetate fraction of "kamena-mena" (*Clerodendrum paniculatum*) leaves [17] ethyl acetate fraction of "senggani" (*Melastoma candidum* D. Don) leaves [23], ethyl acetate fraction of stem bark of "Raghu" (*Dracontomelon dao* (Blanco) Merr) [24], and ethyl acetate fraction of "jackfruit" (*Artocarpus heterophyllus* Lamk) seeds [25]. The strength of the antioxidant activity is due to the high levels of total phenolic and total flavonoid contents found in the medicinal plants. The phenolic and flavonoids compounds are the main phytochemicals responsible for free radical scavenging from plants. Phenolic compounds and flavonoids scavenge free radicals by donating protons or electrons in the hydroxy groups to bind free radicals [26].

Two pattern recognition chemometrics (PCA and CA) were used for the classification and discrimination of the studied plant samples [27]. PCA is a technique used to reduce the multivariate data when there is a correlation between variables. The objects (samples) with similar PCs (principal component) indicated the similar physicochemical properties, so that PCA can be used for grouping among samples. For this reason, PCs are called as latent variables due to its capability to describe the properties of objects (samples) evaluated by PCA. Figure 1A revealed the score plot describing the combination of initial variables (DPPH, ABTS, FRAP, TPC and TFC) and was expressed by the first PC1 and second PC (PC2). Two PCs accounted the data variation of 83.6%, in which PC1 and PC2 contribute to variation of 68.4% and 15.2%, respectively. According to Kumar [28], if the cumulative of two PCs is more than 80%, it has provided sufficient information to describe the existing set of variables. Therefore, using two PCs, the data variations could be described by 2 PCs. The score plot explains the similarity of a sample with other samples based on

PC1 and PC2, which are indicated by the dots that are close to each other. The closer the two points are, the more similar the two samples. Based on the results, the extracts and fractions of several medicinal plants were grouped into four groups based on their similar physicochemical properties (antioxidant activity, TPC, and TFC). One way to determine how many PCs are needed to reduce the number of variables being analyzed is to pay attention to the values of the scree plot [29]. Scree plot values are eigenvalue data obtained from PCA analysis results. There is a significant decrease of eigen values corresponding to PC1 and PC2, and not significant decrease from PC2 to PC3 (Figure 1B), therefore PC1 and PC2 are chosen for developing score plot during PCA.

Table 1. Antioxidant activity, phenolic content, and flavonoid content of several medical plants.

Part of plant	Sample	IC_{50} value (µg/mL)			Phenolic content (µg/mL)	Flavonoid content (µg/mL)
		FRAP	DPPH	ABTS		
Diplazium esculentum	Methanol extract	45.21±0.17	35.65±0.54	53.31±0.51	23.66±0.28	33.11±0.19
	n-hexane fraction	35.09±0.18	21.96±0.53	16.05±0.33	35.66±1.04	50.11±1.07
	Ethyl acetate fraction	24.86±0.44	12.25±0.34	10.97±0.23	47.66±0.76	59.78±0.38
	Water fraction	31.43±0.52	27.38±0.69	40.89±0.11	10.66±1.04	22.88±0.38
Chromolaena odorata L.	Methanol extract	24.86±0.26	31.06±0.62	35.01±0.55	14.63±1.04	24.32±0.62
	n-hexane fraction	30.97±0.37	21.18±1.05	47.88±0.59	26.96±0.64	25.53±1.92
	Ethyl acetate fraction	21.61±0.43	11.24±0.74	31.79±0.63	36.09±0.63	51.28±1.13
	Water fraction	34.74±0.21	18.35±0.27	42.01±0.41	10.93±0.42	18.90±0.81
Ipomea batatas L.	Methanol extract	47.41±0.19	42.15±0.58	19.09±1.31	15.79±0.65	26.52±0.74
	n-hexane fraction	33.35±0.18	13.76±0.63	14.23±0.31	36.98±0.76	35.72±0.76
	Ethyl acetate fraction	33.83±0.43	13.33±0.58	18.14±0.21	62.28±1.87	44.54±0.89
	Water fraction	45.42±0.18	23.57±0.44	31.21±0.06	11.37±0.37	18.04±0.34
Manihot esculenta	Methanol extract	32.41±0.25	41.15±0.95	48.81±0.21	18.88±0.29	27.91±0.21
	n-hexane fraction	25.93±0.03	15.07±0.88	27.86±0.23	33.45±0.79	30.59±0.69
	Ethyl acetate fraction	22.39±0.47	12.08±0.85	29.69±0.31	41.83±0.89	49.60±0.85
	Water fraction	35.57±0.07	21.67±0.56	38.74±0.28	13.58±0.67	26.13±0.69
Vernonia amygdalina Del	Methanol extract	36.23±0.09	29.92±0.34	44.39±0.23	23.37±1.93	35.07±2.53
	n-hexane fraction	34.48±0.07	15.91±0.49	16.31±2.16	40.93±0.42	54.56±1.58
	Ethyl acetate fraction	26.07±0.35	13.17±0.51	14.02±0.17	50.80±1.19	65.16±0.38
	Water fraction	66.81±0.53	36.93±0.61	47.63±0.45	14.43±1.14	17.04±0.47

FRAP, ferry reduction antioxidant power; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)

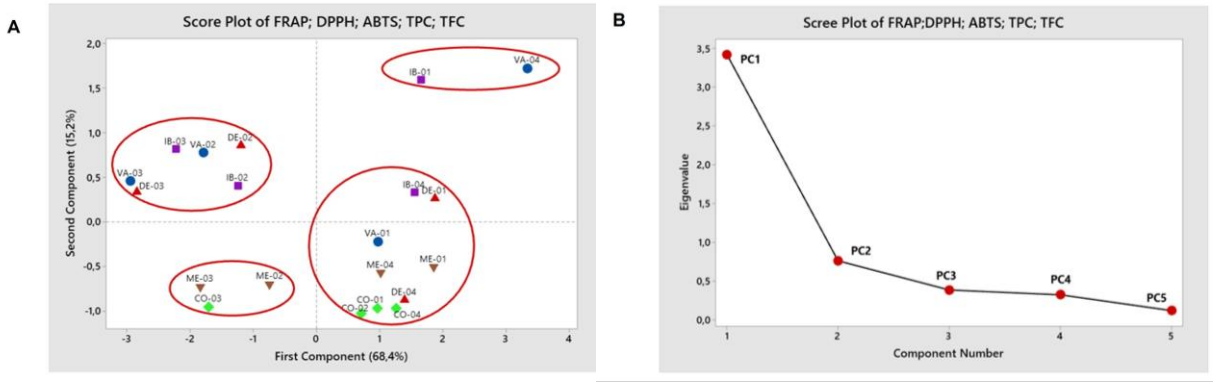

Figure 1. Score plot of principal component analysis (PCA) described by the first principal component (PC1) and second principal component (PC2) of several medicinal plants [a] along with scree plot of PCA method samples [b]. DE = *Diplazium esculentum* leaves; CO = *Chromolaena odorata* leaves; IB = *Ipomea batatas* leaves, ME = *Manihot esculenta* leaves; VA = *Vernonia amygdalina* leaves; 01 = methanol extract; 02 = n-hexane fraction; 03 = ethyl acetate fraction; and 04 = water fraction.

To correlate antioxidant activities (IC_{50} values of DPPH, ABTS and FRAP assays) with TPC and TFC, the chemometrics approach based on loading plot of PCA was used. Figure 2 showed loading plot curve of PCA for developing the relationship between IC_{50} values of FRAP, DPPH, ABTS and TPC, TFC. The loading plot illustrates the power of the variable affecting the PC value, which is described as a vector [30]. If the two

vectors form an angle of less than 90°, it indicates that the two variables are positively correlated. If the two vectors form an angle of more than 90°, it indicates that the two variables are negatively correlated. Meanwhile, if the two variables form a 90° angle, it indicates that the two variables are not correlated. If two vectors formed angle 180°, it indicates that there is no correlation between two variables [12]. TPC and TFC negatively correlated with IC_{50} values (FRAP, DPPH, and ABTS radical), with different correlation strengths, indicating that the higher TPC and TFC concentrations, the lower the IC_{50} values, and the higher the antioxidant activities. TPC and TFC gave a weak correlation to IC_{50} values obtained from FRAP assay, a moderate correlation to IC50 values obtained from DPPH assay, and the strongest correlation to IC_{50} values of ABTS assay.

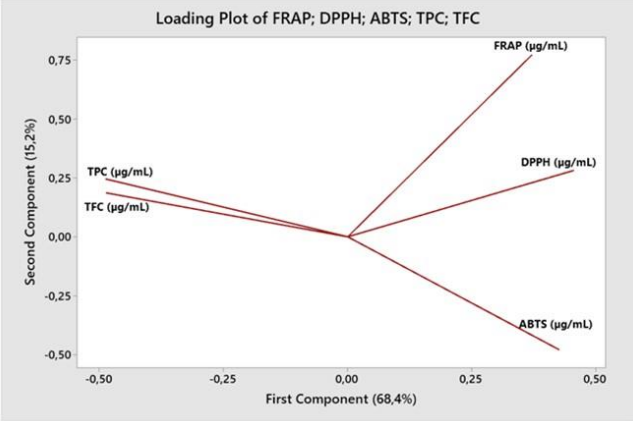

Figure 2. The loading plot curve of principal component analysis (PCA) for the relationship between IC_{50} values of 2,2-diphenyl-1-pikrihydrazyl method (DPPH assay); 2,2'-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Ferric Reduction Antioxidant Power (FRAP) assays with total phenolics content (TPC) and total flavonoid contents (TFC).

The chemometrics of cluster analysis (CA) was then used for making the cluster among the studied medicinal plants, based on the Euclidean distance which is measured based on the closest point of an object (Single linkage) [31]. Figure 3 shows the dendrogram using the single linkage method. The analysis showed that several medicinal plants extracted with different solvents were grouped into four major groups, namely group 1 containing methanol extract (ME) of *D. esculentu,* ME of *V. amygdalina* leaves, ME of *M. esculenta* leaves, water fraction (WF) of *D. esculentum* leaves, WF of M. esculenta leaves, WF of *C. odorata* leaves, WF of *I. batatas* leaves, ME of *C. odorata* leaves, and *n*-hexane fraction (HF) of *C. odorata* leaves. Group 2 consisted of ME of *I. batatas* leaves and WF of *V. amygdalina* leaves. Group 3 consisted of HF of D. *esculentum* leaves, HF of V. *amygdalina* leaves, the ethyl acetate fraction (EF) of C. odorata leaves, EF of M. *esculenta* leaves, HF of I. *batatas* leaves, and HF of M. *esculenta* leaves. Finally, the members of group 4 were EF of D. *esculentum* leaves, EF of *V. amygdalina* leaves, and EF of I. *batatas* leaves**.**

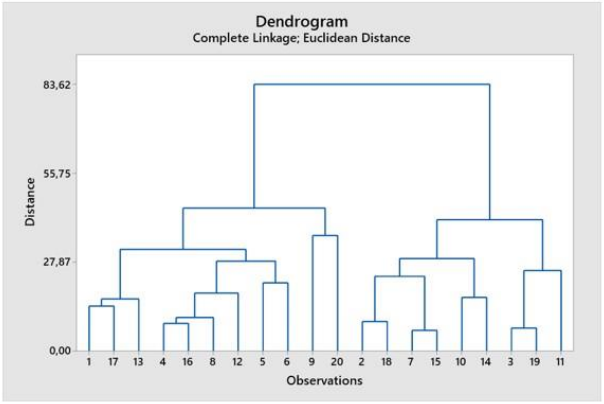

Figure 3. Dendrogram of extracts and fractions of several medicinal plants obtained from cluster analysis. (1) = DE-01; (2) = DE-02; (3) = DE-03; (4) = DE-04; (5) = CO-01; (6) = CO-02; (7) = CO-03; (8) = CO-04; (9) = IB-01; (10) = IB-02; (11) = IB-03; (12) = IB-04; (13) = ME-01; (14) = ME-02; (15) = ME-03; (16) = ME-04; (17) = VA-01; (18) = VA-02; (19) = VA-03; (20) = VA-04. DE = *Diplazium esculentum* leaves; CO = *Chromolaena odorata* leaves; IB = *Ipomea batatas* leaves, ME **=** *Manihot esculenta* leaves; VA **=** *Vernonia amygdalina* leaves; 01 = methanol extract; 02 = n-hexane fraction; 03 = ethyl acetate fraction; and 04 = water fraction.

2.1. Characterization of FTIR spectra of studied medicinal plants

FTIR spectra are fingerprinting in nature, therefore FTIR spectra are widely used for the characterization of medicinal plants. Each peak indicates a functional group present in the extract of studied medicinal plants [32,33]. The functional groups and vibrational modes were presented in the samples of selected medicinal plants. The functional groups represented in each peak and shoulders in FTIR spectra correlate with the chemical compounds present in the studied samples of medicinal plants from Southeast Sulawesi. The functional groups present in the samples provide an overview of the composition of the metabolites contained in the plant samples [34]. The FTIR spectral pattern in Figure 4 shows an almost identical absorption pattern from all plant samples. However, it shows different intensities, especially the fingerprint regions (1500 – 700 cm^{-1}). The peaks at wavenumbers (1/λ) 2922 cm^{-1} (H3) and 2852 cm^{-1} (H4) indicated the presence of symmetric and asymmetric C-H of methylene (CH_2) and methyl (CH_3) groups, respectively. The strong absorption in these regions suggests the presence of many aliphatic chains, especially those bound to triglycerol [35]. The strong peak at 1/λ 1735 cm^{-1} indicated the presence of C=O (carbonyl) stretching of the saturated aliphatic ester groups of lipid biomolecules. This characteristic peak is further strengthened by the absorption of functional groups in the fingerprint region, namely absorption at 1/λ 1459 cm^{-1} (H6) and 1376 cm^{-1}(H7), indicating the presence of C-H bending hydrocarbons, while the peaks at 1/λ 1166 cm^{-1} (H8) and 1069 cm^{-1} (H9) indicated C-O vibrations from ester-linked to glycerol. Meanwhile, absorption at 720 cm^{-1} (H10) indicates the presence of strong vibrations $(CH_2)n$ from the hydrocarbon chain [36].

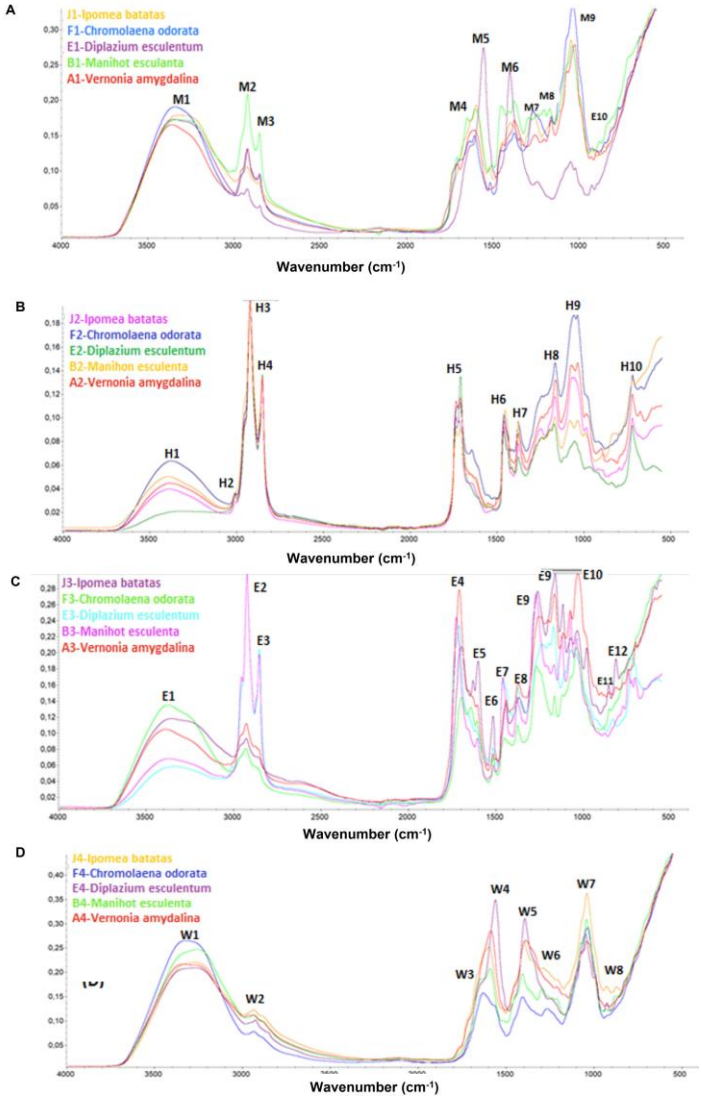

Fig. 4. The overlay of FTIR spectra of (A) = methanolic extract; (B) = *n*-hexane fraction; (C) = ethyl acetate fraction; and (D) = water fraction from several studied medicinal plants.

Partial least squares discriminant analysis (PLS-DA) was employed to choose the variables of FTIR spectral regions used for prediction models for antioxidant activities [37]. The discriminant features identified by PLS-DA were selected based on the important variables in the projection score (VIP). The latter estimates the contribution of each variable to the model, and features with a VIP score > 1 are considered important discriminants. Thus, a total of 8 and 5 features in the multiple medicinal plant leaf data were selected as discriminatory functional groups, as shown in Figure 5. In the leaves of *C. odorata*, the functional groups having the strong contribution are asymmetric C-H and C-O stretching, which appear at 1/λ 2923 (alkane), 1256 cm^{-1}, and 1025 cm^{-1} (alcohol or phenol). Furthermore, for *D. esculentu*m leaves, the functional group with the strongest contribution was asymmetric C-H which appeared at 2923 cm^{-1} (alkanes), and the weakest contributions were C-O stretching (alcohol) and N-H stretching (amines). The functional groups contained in *I. batatas* leaves contribute from strong to weak. The functional groups that make a strong contribution are the C-O group (secondary alcohol) at 1/λ 1161 cm^{-1}, and the weakest is C-C=C stretching (aromatic) at 1/λ 1445 cm^{-1}. The functional groups that make the strongest contribution in *M. esculenta* leaves appear at 1/λ 1256 cm^{-1}, indicating the presence of C-O stretching alcohols and esters, 1/λ 1161 cm^{-1} (C-O stretching secondary alcohols), 1375 cm^{-1} (C-H bending alkanes), and 10-25 cm^{-1} (C-O stretching alcohol). The FTIR spectral regions offering the strong contribution to the characterization of the studied medicinal plants were further optimized to build the prediction models of antioxidant activities.

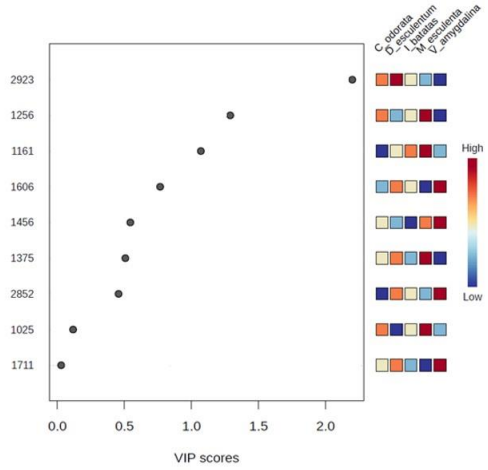

Figure 5. The most important discriminant functional group (identified by PLS-DA) ranked by variable importance in projection (VIP) from several leaf samples are indicated with a colour code scaled from blue (low) to red (high).

2.2. Prediction of antioxidant activities using FTIR spectra and PLSR

With the development of multivariate calibration such as PLSR, it is possible to make the prediction of certain biological activities including antioxidant activities with the chemical responses obtained from chemical measurement (FTIR spectra). PLSR using the FTIR absorbance values as a variable calibration is optimized and used to predict the antioxidant activity by scavenging DPPH radicals during modeling. Some wavenumbers regions (3600 – 650, 3000 – 650, 2960 – 800, 1800 – 650, 1300 – 700, and the combined wavenumbers of 3050 – 2800 and 1790 – 650 cm^{-1}) along with FTIR spectral treatments (normal, first derivative and second derivative) with treatment spectra optimization namely normal) are optimized in terms of its accuracy and precision of the model. Table 2 shows the optimization results of PLSR expressed by R^2-values (for accuracy) along with RMSEC (root mean square error of calibrations), and RMSEP (root mean square error of predictions) for model precision. The selection of the prediction model was based on the capability of PLSR to provide the highest R^2 value with low values RMSEC and RMSEP values. Thus, the first derivative FTIR spectra at wavenumbers 3600 - 650 were selected for the prediction model of DPPH antioxidant assay in selected medicinal plants due to its capability to provide the highest R^2 values in calibration model (0.9974) and prediction model R^2 (0.9983) with low RMSEC value (0.706) and RMSEP value (1.05), as shown in Figure 6A. From the residual analysis, the differences of all points lie above and below zero indicating that systematic errors are not occur. Therefore, FTIR spectroscopy combined with chemometrics can be used to predict antioxidant activity (DPPH radical assay, in this study). It is interesting in the next study to correlate the responses of FTIR spectra with the results of other antioxidant assays.

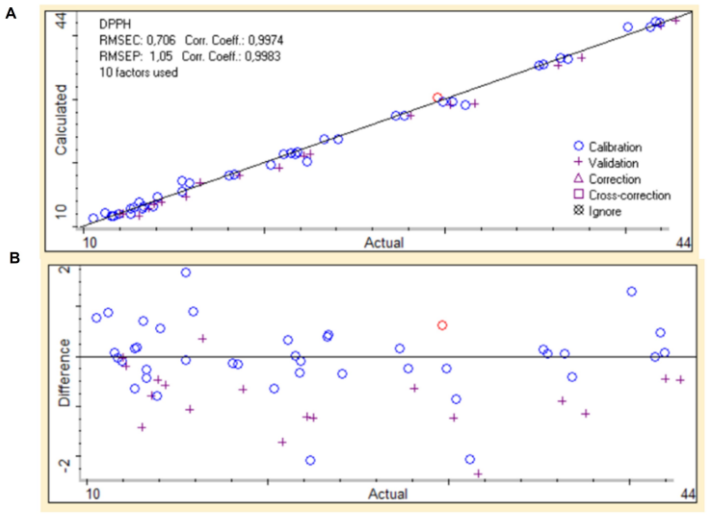

Figure 6. PLS calibration model (a) and residual analysis (b) for prediction of DPPH scavenging activity in several medical plants. The *x*-axis showed the actual IC_{50} values of radical scavenging activity in several medical plants; the *y*-axis showed the predicted radical scavenging activity using FTIR spectral responses at selected condition, as in Table 2.

3. CONCLUSION

The ethyl acetate fraction of medicinal plants provided the strongest antioxidant activities as determined using DPPH, ABTS and FRAP assays with high levels of total phenolic and flavonoids. Loading plot PCA revealed that there is good correlation existed between antioxidant activities and TPC and TFC. FTIR spectroscopy using first derivative treatment at wavenumbers of 3600 – 650 cm^{-1} combined with a multivariate calibration of PLSR has been successful used to predict DPPH antioxidant capacity of medicinal plant extracts. FTIR spectroscopy is taken into account as an alternative method of determining antioxidant activity with the main advantages of fast, simple, selective, valid, and environmentally friendly

4. MATERIALS AND METHODS

4.1. Materials

The medicinal plants were obtained from the Southeast Sulawesi region, Indonesia. Plant samples were authenticated at the Biology Laboratory of the Faculty of Teaching and Education, Halu Oleo University, under the supervision of Mrs. Asmawati Munir, M.Sc. DPPH and ABTS were purchased from Sigma (Aldrich, St. Louis, MO). The other solvents and reagents used were of analytical grade which are obtained from E. Merck (Darmstadt, Germany).

4.2. Extraction and fractionation

Eight hundred grams of each medicinal plant leaf was extracted using the maceration method for 3 x 24 hours at room temperature, and every 24 hours, a new solvent was filtered and replaced. Furthermore, the resulting filtrate was concentrated using a rotary evaporator at 40°C to get the corresponding extract. The obtained extract was then fractionated using liquid-liquid extraction (LLE) method. As much as 30 grams of sample extract was dissolved in distilled water, fractionated with *n*-hexane, then fractionated using ethyl acetate. The fraction results were concentrated using a rotary evaporator at 40°C.

4.3. DPPH radical scavenging assay

DPPH radical scavenging test was carried out by Zubaydah et al. [25]by taking 1.0 mL of a plant extract sample and then added to a 0.04 mM DPPH solution, and finally dissolved in methanol up to 10 mL. The mixture was incubated for 30 min in a dark room. Then, the absorbance was measured at 515 nm. The percentage of DPPH scavenging activity:

$$\text{Percentage (\%) of DPPH radical scavenging activity} = \frac{Abs\ control - Abs\ sample}{Abs\ control} x\ 100\%$$

4.4. Determination of the antioxidant activity of the FRAP method

The reducing ability of plant extracts was estimated using the iron-reducing antioxidant strength test (FRAP) according to Sharma and Vig [24] with slight modifications. FRAP reagent was prepared by mixing acetate buffer (200 mM; pH 6.6) with 1.0 mM of 1% $K_3(CN)_6$ solution, and added with 1 mL of 10% trichloroacetic acid and centrifuged at 3000 rpm for 10 minutes. A-1.0 mL of the supernatant was taken, added with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. Then the absorption was measured spectrophotometrically at 582 nm.

4.5. ABTS cation radical scavenging activity

ABTS assay was carried out according to Yamin et al. [17] as follows: the working solution of fresh $ATBS^{\cdot+}$ was prepared by dissolving 18 mg ABTS in potassium persulfate solution (5 mL; 2.45 mM), thoroughly mixed and stored in a dark place (room temperature; 12 –16 hours). A-1.0 mL of sample was added with 3 mL of methanol and 1 mL of ABTS stock solution. Then, the solution was homogenized, incubated for 10 min at room temperature. The absorbance was measured at a wavelength of 734 nm. Ascorbic acid is used as standard. The percentage of inhibitory activity was calculated using [(A0 – A1)/A0] × 100, where A0 is the absorbance of the control and A1 is the absorbance of the extract or standard. The inhibition curve is generated, and IC_{50} value is calculated. L-(+)-ascorbic acid (Vitamin C) with a final concentration of 0–5 µg/ml was used as a standard antioxidant.

4.6. Measurement of total phenolic content (TPC)

Determination of TPC in the samples was determined using the Folin-Ciocalteu method [39]. The reaction mixture consisted of 1.0 mL of sample, 0.4 ml of Folin-Ciocalteu reagent (Fluka, Steinheim, Switzerland), and 4 mL of 7% sodium carbonate solution (added 8 min after the addition Folin-Ciocalteu reagent). Then, the volume was made to 10 mL. After that, the flask was left to stand for 30 min. The absorbance of the blue sample was measured at 750 nm using a UV-Vis spectrophotometer. Results are expressed as mg gallic acid equivalents per 100-gram dry sample (mg GAE/100 g) compared to the calibration curve generated with standard gallic acid.

4.7. Measurement of total flavonoid content (TFC)

Determination of TFC in the evaluated samples was determined using the Aluminum chloride colorimetric method [40]. The reaction mixture consisted of 1.0 mL sample, 3 mL methanol, 0.2 mL of 10% aluminum chloride, and 0.3 mL of 1 M potassium acetate. Then, the volume was made up to 10 mL with methanol. After that, the flask was left to stand for 30 min. The absorbance was measured at 515 nm using a UV-Vis spectrophotometer. Results are expressed as the equivalent of mg quercetin per 100-gram dry sample (mg QE/100 g) compared to the calibration curve generated with standard of quercetin.

4.8. FTIR spectral measurements

FTIR spectra measurements were carried out by attenuated total reflectance-FTIR spectrometer in the middle IR region (4000 – 650 cm^{-1}) with 32 scans and resolution of 8 cm^{-1}. All spectra were measured after measuring the background spectra. The results of sample measurement will be subtracted automatically with the background spectra by computer software to obtain the analyzed sample spectra. FTIR spectra are recorded as absorbance values in each data.

4.9. Chemometric analysis

The measured data were calculated for the average value and standard deviation (SD) using Microsoft Excel. The absorbance values of the FTIR spectrum were used as a variable to predict the antioxidant activities of leaf extracts of several medicinal plants from Southeast Sulawesi, Indonesia. Principal component analysis (PCA) and cluster analysis (CA) were used for pattern recognitions of studied medicinal plants. PLSR was used to predict DPPH radical scavenging using a variable absorbance value of FTIR spectra. Chemometric analyses (PCA, CA, and PLS) were carried out using Minitab® 19 (Minitab Inc., State College, PA) and MetaboAnalyst (https://www.metaboanalyst.ca).

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