

Prediction of Antioxidant Capacity of Thai Indigenous Plant Extracts by Proton Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Antioxidant capacities of the ethanol extracts from 28 Thai indigenous plants were determined by FCRC, DPPH, ABTS and FRAP assays. Proton nuclear magnetic resonance spectra of the extracts dissolved in D₂O were measured at 300 MHz. The antioxidant capacity and integrated peaks of nuclear magnetic resonance (NMR) spectra were analyzed by partial least square (PLS) regression. Linear correlation was found between the actual and predicted antioxidant capacities of all assays with R² more than 0.84 for calibration models and more than 0.54 for cross-validation models. However, these values were lower than those using infrared (IR) spectroscopy in our previous study. The lower efficiency of NMR-PLS regression might be due to the integrated NMR spectra having lower resolution than the whole IR spectra. Therefore, full NMR spectra should be further investigated for predicting the antioxidant capacity or other biochemical properties of plant extracts.

Keywords: Thai plants, Antioxidant capacity, Nuclear magnetic resonance spectroscopy, Partial least square regression

INTRODUCTION

Plants are important sources of phytochemicals with various biological activities, including antioxidant activity, which has been studied extensively. Antioxidants inhibit free radicals by hydrogen atom transfer or electron transfer. Various assays should be conducted to evaluate the total antioxidant capacity of a sample (Karadag et al., 2009). Commonly used antioxidant assays include 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferric reducing antioxidant power (FRAP),

and oxygen radical absorbance capacity (ORAC) assays. ORAC assay measures antioxidant capacity based on hydrogen atom transfer, while DPPH, ABTS and FRAP assays are based on electron transfer (Huang et al., 2005). Folin-Ciocalteu reagent, formerly used for determination of total phenolic content, reacts with any reducing species in a sample by electron transfer; this method is called Folin-Ciocalteu reducing capacity (FCRC) (Magalhães et al., 2010). However, it is time-consuming and expensive to perform all of these assays.

Infrared (IR) spectroscopy and multivariate data analysis, e.g., partial least square regression (PLS) and cluster analysis, has been applied for fast and non-destructive quality control of agricultural commodities and food products (Sun, 2009). IR spectroscopy, in combination with PLS regression, has effectively predicted the chemical composition and antioxidant activity of food products (Lam et al., 2005; Versari et al., 2010; Leopold et al., 2011). In measurement of IR spectrum, only KBr is required for sample preparation in KBr pellet technique and no reagent is required for horizontal attenuated total reflectance technique (Meissl et al., 2007). Therefore, IR spectroscopy with multivariate analysis can cost effectively predict the antioxidant capacity of food products; many antioxidant assays can be predicted by a single IR spectrum (Lu et al., 2011).

In organic chemistry, IR spectroscopy is used in combination with nuclear magnetic resonance (NMR) spectroscopy for the structural elucidation of compounds (Lampman et al., 2009). While both techniques provide similar information, NMR spectroscopy is higher resolution (Edwards, 2006). Therefore, NMR and IR spectroscopy should theoretically be used for multivariate analysis of antioxidant activity of food samples.

The objective of this study was to apply NMR spectroscopy to predict the antioxidant capacities of plant extracts that were determined by ET-based assays, including FCRC, ABTS, DPPH and FRAP assays. In this way, four antioxidant capacity values of plant extracts were determined by a single NMR measurement, reducing sample preparation, chemical reagents and the time required for conducting all antioxidant assays.

MATERIALS AND METHODS

Raw materials

Plants were collected from local markets and a farm in Chiang Mai, Thailand in August-September, 2012 (Table 1). A wide range of plants was randomly selected in order to obtain a wide range of antioxidant capacities for making the regression model. The moisture content of the plants was determined by the AOAC method (AOAC, 2000).

Analytical grade ethanol for extraction was obtained from J.T. Baker (Coopersburg, PA, USA). All other analytical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table 1. Antioxidant capacities of freeze-dried Thai indigenous plant extracts.

No.	Common name	Scientific name	Moisture (%)	Part	Antioxidant capacities ^a			
					FCRC ^b	DPPH ^c	ABTS ^c	FRAP ^c
1	Mango cv. Talapnak	<i>Mangifera indica</i> L. cv. Talapnak	81.34±0.35	Leaves	45.23±1.06	342.21±21.40	316.87±7.57	282.84±0.94
2	Mango cv. Chokanan	<i>Mangifera indica</i> L. cv. Chokanan	79.08±0.03	Leaves	43.80±1.03	355.65±11.46	310.67±7.54	272.51±2.84
3	Mango cv. Namdokmai	<i>Mangifera indica</i> L. cv. Namdokmai	78.90±0.30	Leaves	50.46±0.20	405.52±13.01	358.18±12.34	313.40±2.46
4	White fig	<i>Ficus lacor</i> Buch.	80.98±0.21	Leaves	51.78±0.80	342.64±15.83	192.23±4.41	247.95±9.39
5	Olive	<i>Spondias pinnata</i> Kurz.	81.54±0.23	Leaves	37.05±0.31	305.35±15.02	174.12±5.52	234.04±7.52
6	Yellow cow wood	<i>Cratogeomys cochinchinense</i> (Lour.) Blume	82.20±0.31	Leaves	23.81±0.27	117.58±6.23	71.73±2.45	75.16±2.21
7	Lemon balm	<i>Melissa officinalis</i> L.	83.14±0.13	Leaves	32.14±0.16	187.40±13.02	149.70±2.61	202.56±5.47
8	Water mimosa	<i>Neptunia oleracea</i> Lour.	79.29±0.30	Leaves	31.34±0.48	248.54±11.36	179.61±2.16	154.70±2.39
9	Chiangda	<i>Gynema inodorum</i> Decne.	83.56±0.30	Leaves	17.14±0.38	9.61±7.49	31.51±3.74	28.09±1.02
10	White mugwort	<i>Artemisia lactiflora</i> Wall.		Leaves	15.93±0.40	61.64±16.33	64.80±2.93	61.60±1.93
11	White leadtree	<i>Leucaena glauca</i> Benth.	78.64±0.30	Leaves	49.04±0.34	318.36±7.89	265.69±9.71	249.36±6.45
12	Paem	<i>Acanthopanax trifoliatum</i> Merr.	79.85±0.37	Leaves	13.75±0.07	65.55±9.22	56.61±1.91	59.48±1.59
13	Vietnamese coriander	<i>Polygonum odoratum</i> Lour.	71.74±0.12	Leaves	43.93±0.47	320.09±15.83	220.07±17.87	211.99±4.07
14	Clove basil	<i>Ocimum gratissimum</i> L.	80.12±1.12	Leaves	20.32±0.26	95.47±5.01	103.58±4.52	109.46±1.89
15	Climbing wattle	<i>Acacia pennata</i> (L.) Willd.	81.86±0.11	Leaves	17.96±0.28	26.09±5.75	12.80±1.10	12.34±0.15
16	Bamboo grass	<i>Tiliacora triandra</i> Diels	78.17±0.35	Leaves	13.66±0.26	121.78±4.37	49.08±0.71	61.41±0.38
17	Holy basil	<i>Ocimum sanctum</i> L.	83.51±0.37	Leaves	17.08±0.09	72.05±25.22	76.27±1.61	102.27±1.68
18	Gooseweed	<i>Sphenoclea zeylanica</i> Gaertn.	84.13±0.25	Leaves	12.42±0.10	38.23±9.96	34.11±1.14	39.17±0.64

Table 1. Antioxidant capacities of freeze-dried Thai indigenous vegetable extracts (Continued).

No.	Common name	Scientific name	Moisture (%)	Part	Antioxidant capacities ^a			
					FCRC ^b	DPPH ^c	ABTS ^c	FRAP ^c
19	Lemon basil	<i>Ocimum × citriodorum</i>	83.44±0.20	Leaves	19.01±0.25	85.93±6.79	85.68±2.53	102.18±1.88
20	Malabar	<i>Basella alba</i> L.	90.99±0.14	Flower	8.84±0.15	7.44±11.62	8.50±1.58	11.33±0.53
21	Drumstick tree	<i>Moringa oleifera</i> Lam.	78.47±0.14	Leaves	11.23±0.35	27.39±1.66	28.46±1.46	32.90±1.50
22	Vegetable fern	<i>Diplazium esculentum</i> (Retz.) Swartz	86.24±0.48	Leaves	14.17±0.20	19.58±7.41	16.28±0.57	15.59±0.43
23	Latherleaf	<i>Colubrina asiatica</i> (L.) Brongn.	76.28±0.63	Leaves	12.47±0.20	55.14±6.70	23.68±1.22	29.08±1.26
24	Paracress	<i>Spilanthes acmella</i> Murr.	84.49±0.15	Leaves	9.94±0.14	38.66±9.43	32.33±0.98	35.22±1.45
25	Wild betel	<i>Piper sarmentosum</i> Roxb. Ex Hunter	82.43±0.16	Leaves	10.53±0.08	16.11±10.25	17.30±6.58	24.44±0.16
26	Tonkin jasmine	<i>Telosma minor</i> Craib	88.06±0.44	Flower	6.57±0.08	2.67±8.14	7.85±2.22	9.55±0.58
27	Noni	<i>Morinda citrifolia</i> L.	79.59±0.15	Leaves	8.09±0.10	35.63±6.79	23.39±17.19	18.12±0.55
28	Sano	<i>Sesbania javanica</i> Miq.	88.03±0.17	Flower	4.87±0.18	16.11±11.54	6.22±5.65	9.32±0.26

Note: ^a Means ± standard deviation of at least triplicate analysis. ^b Unit of FCRC was g GAE equivalent/100 g extract. ^c Unit of DPPH, ABTS, FRAP was mmol Trolox equivalent/100 g extract.

Preparation of plant extracts

Edible parts of 28 plants were washed with tap water for about 1 min at room temperature and drained for 1 h. Each plant was extracted with 60% ethanol at a plant-to-solvent ratio of 1:5 (w/v) using a blender (Model MR 4050 CA, Braun, Spain) for 30 s (Prommajak et al., 2014). Then, the solution was filtered through a double layer of muslin cloth and centrifuged at 2,500 x g (Universal 320 R, Hettich, Tuttlingen, Germany) for 20 min. The supernatant was evaporated by a rotary evaporator (Eyela, Tokyo, Japan), lyophilized by freeze-dryer (Labconco, Kansas City, MO, USA), and ground into powder. The freeze-dried samples were kept at -20°C until use. Extraction was performed in triplicates.

Determination of antioxidant capacities

The dry powder (1 mg) was dissolved in 1 mL of 50% (v/v) ethanol and was then analyzed for antioxidant capacities. Folin-Ciocalteu reducing capacity (FCRC) was analyzed by the modified method of Dudonné et al. (2011). The result was expressed as gram gallic acid equivalent (GAE)/100 g extract.

2,2'-Azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging capacity (RSC) was analyzed according to the method of Re et al. (1999). 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacity assay was determined according to the modified method of Payet et al. (2005). Ferric reducing antioxidant power (FRAP) was determined according to the method of Benzie and Strain (1996). The result was expressed as mmol Trolox equivalent (TE) /100 g extract. Analyses were performed at least in triplicates.

TE value was calculated as the following equation:

$$\text{Antioxidant capacity (mmol TE/100 g extract)} = \frac{A \times V \times D}{W \times 10}$$

where A is TE calculated from standard curve (mM), V is volume of solvent used for dissolving the extract (ml), D is dilution factor, and W is weight of freeze-dried extract (g).

Nuclear magnetic resonance spectroscopy

Plant extract (5 mg) was dissolved in 1 ml of D₂O. Proton NMR spectra were acquired by JNM-LA300 FT NMR system (Jeol, Tokyo, Japan) operated at 300 MHz. Peak integration was performed and the proton contents in each chemical shift were used for statistical modeling.

Statistical analysis

Partial least square (PLS) regression was performed with Unscrambler version 9.7 (CAMO Software AS, Oslo, Norway). Integrated NMR peaks were used as explanatory variables, while antioxidant capacities were used as response variables. Data centering was conducted prior to statistical analysis. Sample outliers were excluded from the model to obtain a higher correlation coefficient and lower prediction error. Leave-one-out cross validation was used for model validation. K-means cluster analysis and data visualization were performed by

R version 2.15.2 (<http://cran.r-project.org/>) with ‘cluster’ package (Maechler et al., 2013).

RESULTS

Antioxidant capacities of plant extracts

Antioxidant capacities of plant extracts are shown in Table 1. According to K-means cluster analysis based on all antioxidant capacity values, the samples were divided into four groups. Only ABTS and FRAP data had non-overlapping value between groups. The first group had high antioxidant capacity, with ABTS values of more than 260 mmol Trolox eq./100 g and FRAP values of more than 249 mmol Trolox eq./100 g. This group included *Leucaena glauca* Benth and three varieties of *Mangifera indica* L. The second group had moderate antioxidant capacity, with ABTS values between 149 and 221 mmol Trolox eq./100 g and FRAP values between 154-248 mmol Trolox eq./100 g. This group included *Ficus lacor* Buch., *Spondias pinnata* Kurz., *Melissa officinalis* L., *Neptunia oleracea* Lour. and *Polygonum odoratum* Lour. The third group had moderately low antioxidant capacity, with ABTS values between 49 and 104 mmol Trolox eq./100 g and FRAP values between 59 and 110 mmol Trolox eq./100 g. This group included *Cratogeomys cochinchinense*, *Artemisia lactiflora*, *Acanthopanax trifoliatum*, *Ocimum gratissimum*, *Tiliacora triandra*, *Ocimum sanctum* and *Ocimum × citriodorum*. The

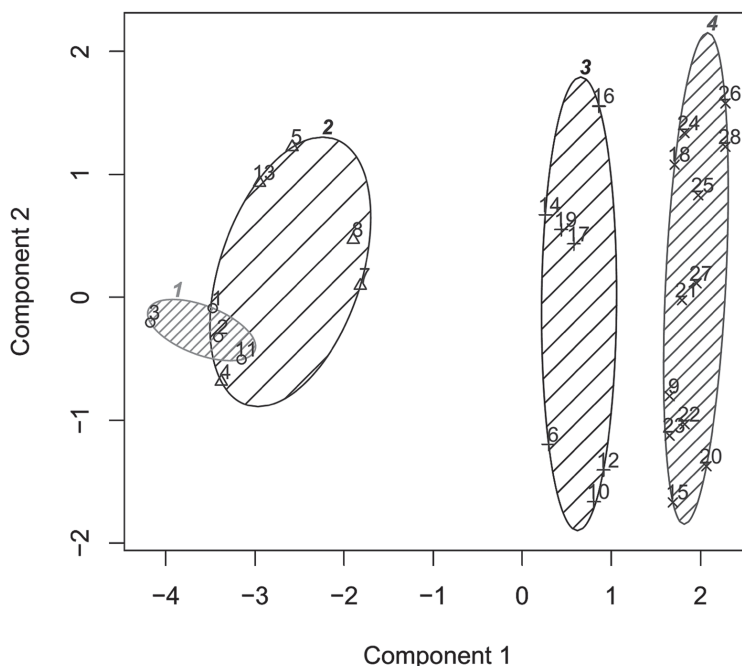


Figure 1. K-means clustering of Thai indigenous plant extracts based on antioxidant capacity.

remaining samples had low antioxidant capacity, with ABTS values lower than 35 mmol Trolox eq./100 g and FRAP values lower than 40 mmol Trolox eq./100 g (Figure 1). Correlations were found between the antioxidant values obtained from the four assays at a significance level less than 0.01 (Figure 2).

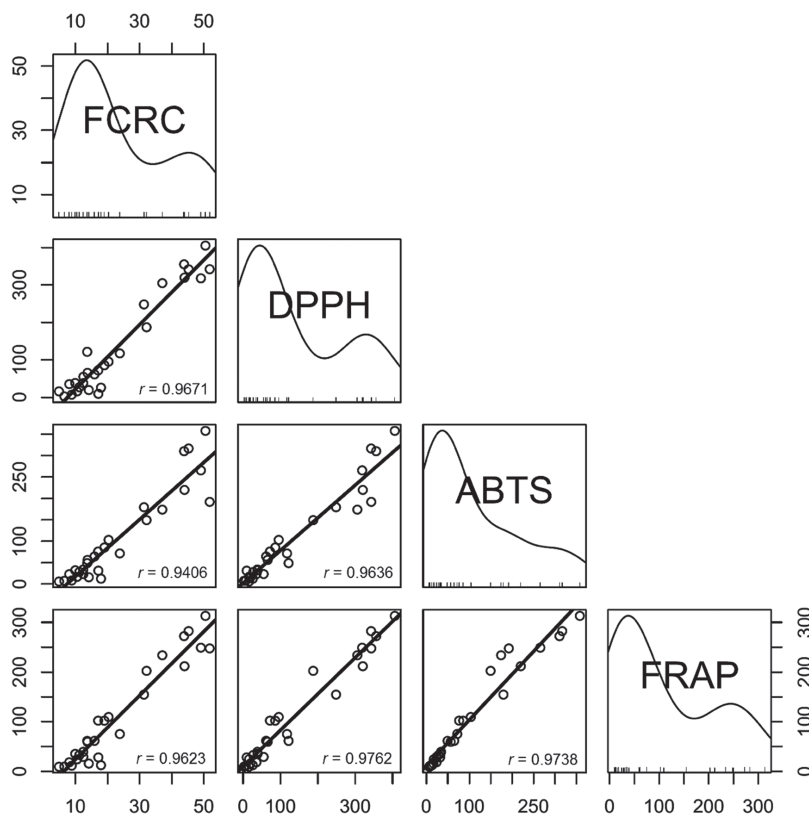


Figure 2. Correlation between FCRC, DPPH, ABTS and FRAP assays with Pearson's correlation coefficient (r).

NMR-PLS regression of antioxidant capacity

The integration data of NMR peaks and antioxidant capacity values were used for PLS regression. Predicted antioxidant capacity was correlated with the measured antioxidant capacity (Figure 3). Coefficients of determination (R^2) were 0.84-0.89 for the calibration models and 0.54-0.68 for the cross-validation models (Table 2). FRAP assay had the highest R^2 of the calibration models, while ABTS assay had the highest R^2 of the validation models. Residual showed the difference between actual and predicted value ($y - \hat{y}$). Water mimosa showed large residuals (Figure 4).

Table 2. Parameters and results of PLS regression.

Antioxidant capacities	Data		No. of samples	No. of PLS components	Calibration			Cross validation		
	Mean	Range			R ²	RMSE	% CE	R ²	RMSE	% CVE
FCRC	20.23	4.87-50.46	24	5	0.8818	4.36	21.55	0.6751	7.60	37.57
DPPH	111.11	2.67-405.52	24	5	0.8424	47.46	42.71	0.5406	83.57	75.21
ABTS	92.69	6.22-358.18	25	5	0.8876	32.06	34.59	0.6842	53.42	57.63
FRAP	98.20	9.32-313.40	25	5	0.8968	30.08	30.63	0.5847	59.27	60.36

Note: Unit of FCRC was g GAE equivalent/100 g. Unit of DPPH, ABTS, FRAP was mmol Trolox equivalent/100 g. RMSE – root mean square error; CE – calibration error; CVE – validation error.

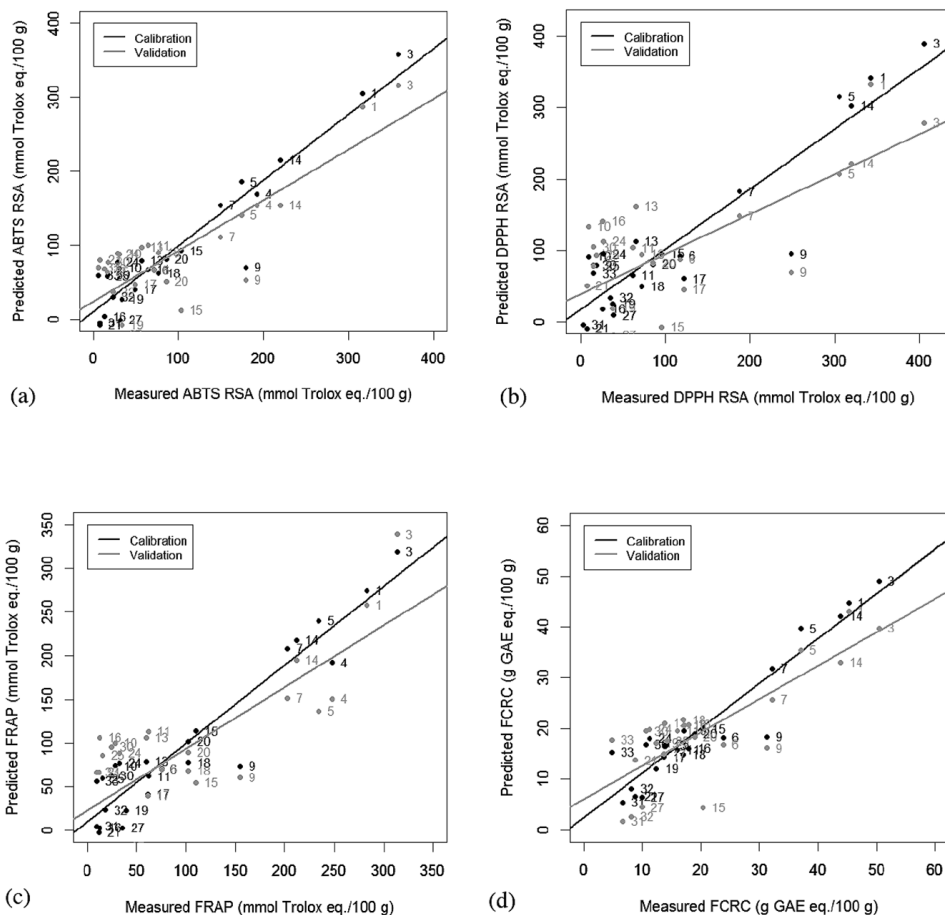


Figure 3. Measured and predicted (a) ABTS, (b) DPPH, (c) FRAP and (d) FCRC values of freeze-dried extract by NMR-PLS regression.

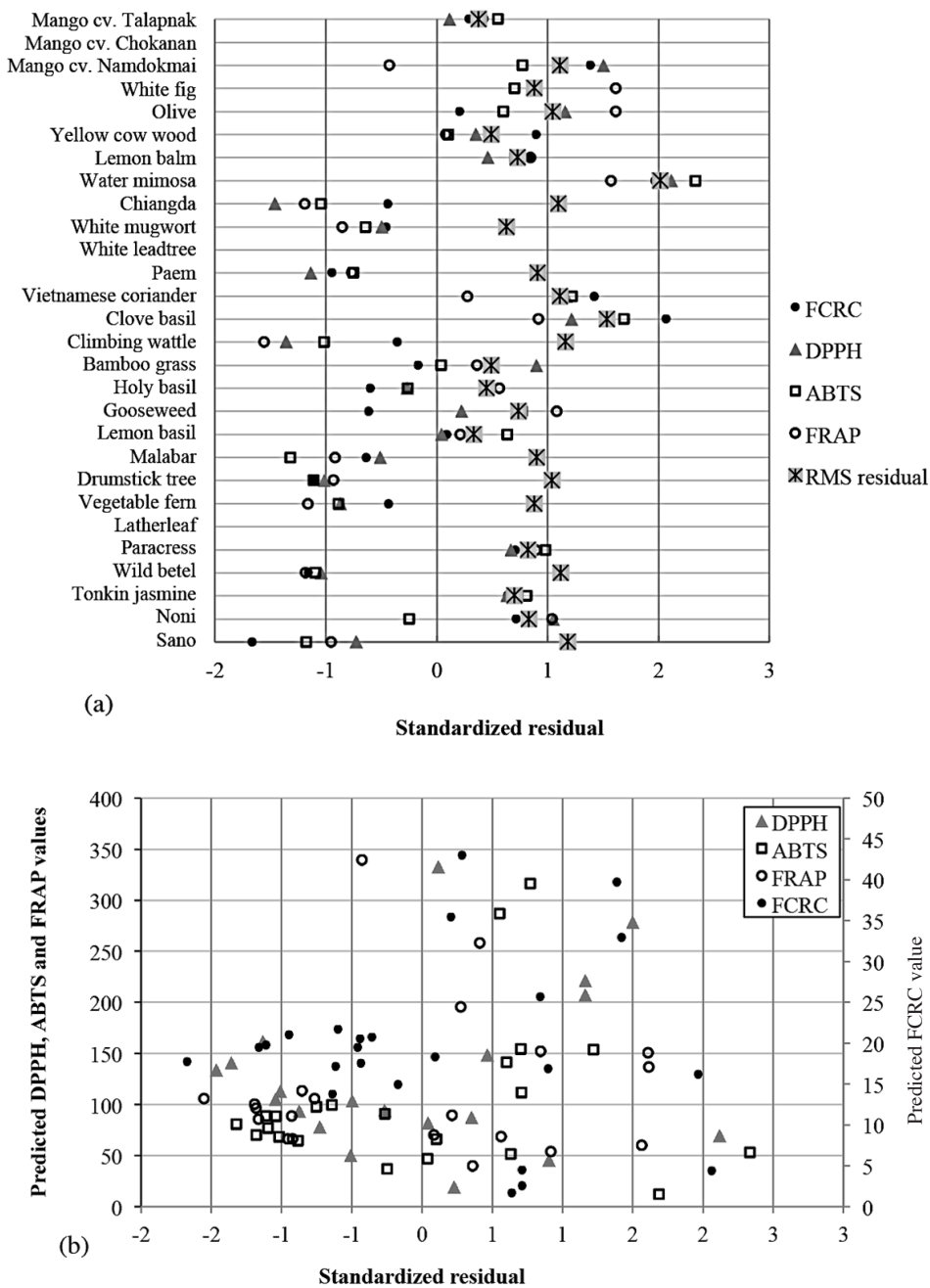


Figure 4. Residual plots of LOO cross-validated NMR-PLS data for (a) individual sample and (b) predicted antioxidant capacity of freeze-dried extract.

The effect of functional groups of bioactive compounds in plant extracts on antioxidant capacity could be determined by regression coefficients of PLS models. The patterns of regression coefficient were similar among all assays (Figure 5). Regression coefficients at the chemical shifts of 1.0, 1.2, 3.5 and 6.9 had large positive values.

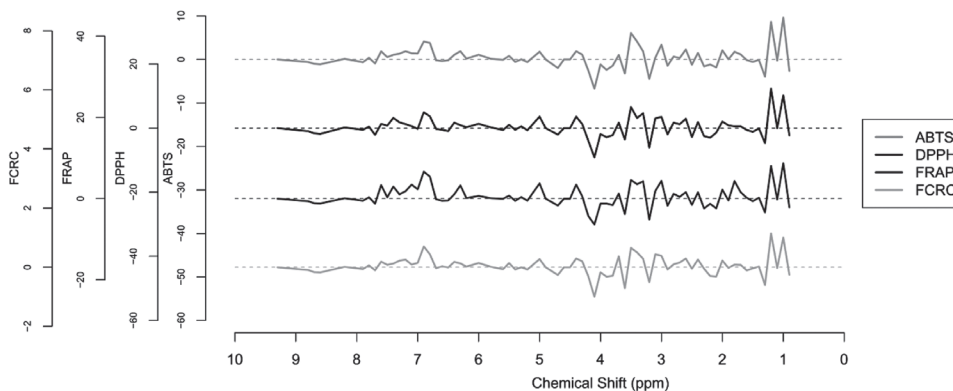


Figure 5. Regression coefficients for the NMR-PLS prediction of antioxidant capacity of (a) liquid and (b) freeze-dried vegetable extracts.

DISCUSSION

The positive correlations between the antioxidant values obtained from four assays may be due to the same electron transfer antioxidant mechanism of the assays (Karadag et al. 2009). Most antioxidant values of the extracts were located in the region of low antioxidant capacity. Frequency plot indicated a similarity between the FCRC, DPPH, and FRAP assays. However, the shape of the frequency plot was different for the ABTS assay. Some samples with a high antioxidant value in three of the assays shifted to the middle region in the ABTS assay. This may be due to different reactions between the assays. The reaction of ABTS radical and phenols was composed of first and secondary reactions between the compounds, which resulted in a rapid reaction. In contrast, the reaction of DPPH radical and phenol is complex and composed of many reversible reactions, which resulted in a different rate of reaction. Moreover, the reaction of DPPH also depended on phenolic compounds (Koleva et al., 2001).

The R^2 values of NMR-PLS models were less than those obtained from our previous study using infrared (IR) spectra ($R^2 > 0.98$ for calibration models and $R^2 > 0.89$ for validation models). This might be because the resolution of integrated NMR data was less than full IR spectra. In partial least square regression, an increasing number of PLS components generally resulted in a higher R^2 value. NMR-PLS had four to five PLS components; this was lower than FTIR-PLS (5-11 components). However, increasing the PLS components in NMR-PLS to a level higher than the reported value caused overfitting and resulted in higher validation errors or lower predictability.

A plot between residual and predicted values showed that the models underestimated or overestimated antioxidant capacity when predicted values were less than 200 mmol TE/100 g. However, when predicted values were higher than this, underestimation mostly occurred (Figure 4b).

A coefficient value higher than zero indicated that the functional groups at the particular chemical shift had a positive effect on the antioxidant capacity of the extract. High positive coefficients occurred at the chemical shift 1.0-1.2 and 3.4-3.5 ppm. The chemical shift around 1 ppm was assigned to the *para*-substituted benzene ring and the chemical shift at 1.2 ppm was assigned to the hydroxyl (OH) group. The chemical shift at 3.4-3.5 ppm was assigned to alkene or alkyne protons that were attached to the hydroxyl group (Lampman et al., 2009).

Minor positive coefficient peaks also occurred at the chemical shift of 6.8-6.9 ppm, which was assigned to the hydroxy-substituted benzene ring. The chemical shift around 7.0-8.0 ppm was assigned to the benzene ring with different substitutions. The presence of a hydroxyl group attached to a benzene ring is a key indicator for antioxidant capacity of phenolic compounds (Arts et al., 2003). The chemical shift at 6.9 and 7.6 ppm was also reported in most flavonoids, which were exhibited by the proton from ring C and B of flavonoids, respectively (Yoon et al., 2011).

On the other hand, a regression coefficient value less than zero indicated that functional groups had a negative effect on antioxidant capacity. The negative coefficients were found at chemical shifts of 1.3, 2.3, 3.2 and 4.1 ppm. The chemical shift at 4.1 could be assigned to the proton in the $-\text{COOCH}_3$ or $-\text{OCH}_2$ groups attached to phenol, which had a steric effect and decreased the antioxidant capacity of the phenolic compounds. Similarly, the negative effect of $-\text{CH}_3$ substitution in phenol was also reflected at the chemical shift at 2.3 ppm (Lampman et al., 2009). These results were confirmed and indicated that phenolic acids and flavonoids were major contributors to antioxidant activity of the samples.

NMR-PLS regression provided models with lower R^2 and higher error when compared with IR-PLS regression. Theoretically, NMR provides more information than IR. However, in this study, peak integration was used instead of full spectrum, which cannot be exported from the computer. Therefore, full NMR spectrum should be further investigated for better predictability and convenience in statistical analysis.

CONCLUSION

NMR spectroscopy was used to predict the antioxidant capacities of plant extracts. NMR-PLS regression had an $R^2 > 0.82$ for calibration models and an $R^2 > 0.52$ for validation models, showing less predictability compared with IR-PLS regression. The lower efficiency of NMR-PLS might due to integrated NMR spectra, which had lower resolution than the whole IR spectra. The coefficients of PLS regression models revealed that the aromatic ring and hydroxyl group were responsible for the antioxidant activity of the plant extracts.

ACKNOWLEDGEMENTS

This work was supported by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0260/2551). C. Pan and S. Kim were supported by a KIST Gangneung Institute Intramural Grant (2Z04220).

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