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Original Article

Antioxidant Activity, HPTLC Fingerprint, and Discriminant Analysis of *Plantago major* Leaves from Diverse Origins in Indonesia

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ABSTRACT

Introduction: *Plantago major* L. *(Plantaginaceae)* is a perennial herb having contribution to the folk medicine all around the world, including Indonesia with wide geographical distribution. Plant materials origin is one factor that significantly influences the quality of herbal medicines. **Materials and Methods:** In this paper, High-Performance Thin Layer Chromatography (HPTLC) method using pattern-oriented approach has been employed to evaluate the quality of *Plantago major* leaves collected from seven origins in Indonesia. To differentiate the antioxidant capacities of those plant materials, the crude extracts were tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH), total phenolics, and total flavonoids assay methods. **Results:** The results showed that radical scavenging activity, total phenolics, and total flavonoids of plant material from seven origins were significantly different. Moreover, HPTLC fingerprints analyzed with chemometrics showed an ability to discriminate the leaves samples from various origins as well as detect chemicals responsible for discrimination. Two models using principal component analysis (PCA) and partial least squares (PLS-DA) were built in chemometrics test. The PCA model was able to describe the studied samples by using four principal components with a value of explained variance of 95%, whereas PLS-DA model accurately classified the leaves samples with prediction ability of 100%. In the PCA, loading plot of the first PC showed that peaks number 10 and 12 are the most important peaks for clustering of the samples. **Conclusions:** *Plantago major* collected from different origins revealed different radical scavenging activity and concentration of total phenolics as well as total flavonoids. HPTLC fingerprints coupled with chemometrics analysis can be used as an alternative to marker-oriented method for the quality control of *Plantago major*.

Key words: Chemometrics, Flavonoids, Herbal medicines, Pattern-oriented, Phenolics, PLS-DA.

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INTRODUCTION

Plantago major L. *(Plantaginaceae)* is a perennial herb that has contributed to the folk medicine all around the world,[1] including Indonesia with wide geographical distribution.[2] Diverse pharmacological effects have been demonstrated for *P. major* such as anti-inflammatory,[3-7] wound healing,[8] antiviral,[9,10] anticancer,[9,11] anticholinesterase,[12] and immunomodulator.[9,13] Additionally, this plant has been investigated for its antioxidant activity.[12,14] The leaves are the most being part used in traditional medicine around the world.[1]

The effect of parts of plant and their growing environment to the chemical compounds of herbal materials have been shown in many studies[15-18] and it is therefore necessary to determine their contents and compositions to assure the consistency of biological activity and to anticipate their potential adverse effects.[19] Marker(s) or biologically active constituents in herbs are commonly employed in quality assessment of herbal medicines.[20] However, this parameter is not always worthy; for instance when the effect of growing condition is required. Since there are tens or even hundreds of constituents, which are slightly different according to their geographical locations, we cannot select only one or several markers as standard. In this case, fingerprint techniques are more recommended for those purposes.[21,22]

A fingerprint is a distinctive profile or pattern of sample which chemically reflects its composition in which as much information as possible is presented and can be developed by spectroscopy, chromatography or electrophoresis methods.[19,22] Chromatographic fingerprint, especially HPTLC-fingerprint, has been widely used in herbal medicines assessment due to its simplicity, rapidity, and economy. It is possible to visually analyze HPTLC-chromatograms; however, this technique is subjective and not quantitative. Moreover, fingerprint chromatograms are complex multivariate data sets which cause difficulty in evaluation of very similar chromatograms. Thus, chemometrics should be taken into consideration. This approach, although more difficult, is based only on objective mathematical methods and treats the chromatogram as a unique signal, without a need to identify and

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interpret the peaks. Therefore, it provides a good possibility for mining more useful chemical information from original-rich data.[22-24]

In our previous work, we established a method to determine the concentration of ursolic acid and oleanolic acid[25] in different plant organs of *P. major*. Nevertheless, the leaves part did not contain oleanolic acid. On the other hand, we observed that leaves extract exhibited the highest DPPH scavenging activity among the other parts. Previous studies supposed that phenolics and flavonoids substances are the most responsible for antioxidant activity of *Plantago.*[26] Therefore, standardization of *P. major* leaves using fingerprints accompanied with the antioxidant capacity seems more appropriate.

As a continuation of our previous work, in this present study we determined radical scavenging activity, total phenolics, and flavonoids contents in *P. major* leaves collected from several origins in Indonesia. We developed also the HPTLC fingerprints combined with chemometrics to differentiate those leaves samples. Although several works both on qualitative and quantitative evaluation of *Plantago* L. have been reported,[27-30] none of them involved comparison between *P. major* from different origins using chemometrics approach. Majority of the present studies focused on the application of marker(s). Therefore, this present study was aimed to (1) determine radical scavenging activity, total phenolics, and total flavonoids of *P. major* leaves extracts collected from various origins in Indonesia, and (2) discriminate *P. major* leaves samples from those origins using HPTLC fingerprint combined with chemometrics method.

MATERIAL AND METHODS

Chemicals

The following chemicals were procured from Sigma (St. Louis, MO, USA): gallic acid, quercetin, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH). Folin-Ciocalteu (FC) reagent was from Fisher Scientific, Leicestershire, U.K. HPTLC plate pre-coated with silica gel 60 F_{254} was from Merck KGaA (Darmstadt, Germany). All other reagents used in this study were of analytical grade.

Plant materials

Plantago major samples were collected from seven origins in Indonesia (Table 1). Whole plant materials were harvested and cleaned with tap water. Leaves were separated from the other organs and used for this study. All samples were dried, ground, and authenticated as described in previous work i.e. by Center of Information and Development of Herbal Medicine, University of Surabaya, Indonesia with certificate number: 1101/D.T/XI/2013.[25]

Extracts preparation

Plantago major leaves extracts were prepared as detailed in our previous study [25]. Briefly, 10 grams of dry ground leaves were soaked three times (24 h each) in 100 ml methanol, subsequently filtered, and evaporated under vacuum to dryness.

Table 1: Geographical origins of *P. major* leaves samples from Indonesia.

Location	Position		Time of collection	Sample code
	Latitude	Longitude		
Lumajang	8°8'0"S	113°13'1"E	October, 2012	L1
Makassar	5°8'37.2"S	119°25'34.8"E	June, 2012	L2
Trawas	7°40'12"S	112°36'36"E	April, 2012	L3
Agam	0°13'15.48"S	100°10'13.2"E	April, 2012	L4
Solok Selatan	1°29'7.0"S	100°10'13.2"E	April, 2012	L5
Lampung	2°11'16.4"S	101°3'40.4"E	June, 2012	L6
Surabaya	7°17'24"S	113°53'33.2"E	August, 2012	L7

DPPH radical scavenging activity

Free radical scavenging assay was carried out according to Galvez et al. with slight modification.[26] A 50 µl of 0.026% DPPH in methanol was added to 100 µl of each dilution of extracts in methanol (total volume 150 µl). After 15 min incubation in darkness, absorbance was measured at 517 nm using a microplate reader. The concentration which produced 50% radical scavenging (IC_{50} value) was extrapolated from the linear regression of concentration versus inhibition (%).[31]

Determination of total phenolics compounds

Determination of the total phenolics content using Folin-Ciocalteu reagent was performed according to the established method,[14, 32] customized for 96-well microplates. Gallic acid, prepared in 8 concentrations ranging from 8 to 1000 µg/ml, was used as a standard. Thirty µl of each extract or standard solution was added to 150 µl of 0.1 mol/l FC reagent and mixed with 120 µl of sodium carbonate (7.5%) after 10 min. Absorbance at 760 nm was read after 2 h. The phenolics concentration was determined by comparison with the standard calibration curve of gallic acid, and the results are presented as a mean value of triplicate tests. The total phenol value was expressed as gram of gallic acid equivalents (GAE) per 100 gram of dry extracts.

Determination of total flavonoids

The aluminum chloride colorimetric method described by Chang *et al.,*[33] adapted for 96-well microplates, was used to determine the total content of flavonoids. Quercetin solution was prepared ranging from 8 to 1000 µg/ml and used as a standard. Thirty microliters of extract or standard solution was diluted with 90 µl of methanol, and 6 µl of 10% $AlCl_3$ (substituted with distilled water in blank), 6 µl of 1 mol/l potassium acetate, and 170 µl of distilled water were added. Absorbance at 415 nm was determined after 30 min. All samples were analyzed in triplicate, and mean values of flavonoid content are expressed as gram of quercetin equivalents (QE) per 100 of dry extracts calculated according to the standard calibration curve.

HPTLC fingerprint analysis

Chromatographic measurement

Each leaves extract was weighted (15.0 mg) in 5 replicates, dissolved in 5.0 ml methanol, filtered using nylon membrane filter (0.45 µm), and then proceeded for HPTLC analysis using the following condition. A Camag TLC system comprising of Linomat 5 sample applicator, twin-through chamber, TLC Plate Heater III, TLC Scanner 3, winCATS 1.2.6 software, and Reprostar 3 (Camag, Muttenz, Switzerland) were used. Chromatography was performed on HPTLC plates 20 x 10 cm, pre-coated with silica gel 60 $F_{254,}$ 0.20 mm layer thickness (Merck, Darmstaadt, Germany) with a 100-µl Camag syringe. Samples were spotted under a flow of nitrogen as 6 mm bands, 15 mm from the left edge, 10 mm from the bottom edge and 10 mm of track distance. Development was carried out in a chamber previously equilibrated (for 20 min at room temperature) with mobile phase, i.e. 30 ml of 1,4 dioxane-xylene-propan-2-ol-12.5% NH_3 (1:2:5:2) and migration distance was 80 mm. The plates were dried under warm air and dipped in 5% sulfuric acid in methanol. Prior to densitometry scanning, TLC plates were dried in fume hood and then heated for 3 min at 120 °C. Fingerprints evaluation were carried out after scanning in absorbance mode at 545 nm; with scanning speed 20 mm/s using slit dimension 6 mm x 0.45 mm and data resolution 100 µm/step. The photographs were captured under white light.

Chemometrics analysis

Before applying the chemometrics technique, chromatographic data arrangement was conducted; i.e. (a) determine the peaks which can

be distinguished from the background noise; (b) describe the peaks intensity (height or area) for all detected peaks, and; (c) present the data in the data matrix.

Principal component analysis (PCA), an exploratory data analysis, was employed as the first step in fingerprints analysis. This method is based on the information available in the fingerprints only. PCA reduces the complexity of the multivariate data set by explaining the correlation amongst a large number of variables in terms of a smaller number of underlying factors (principal components or PCs) without losing much information. The projections of the n objects from the original data on PCs are called the scores plots, whereas the contribution of each original variable to the score is presented by its loading, which detects the variables responsible for the clustering.[19]

The results of PCA were confirmed by a supervised analysis, i.e. partial least squares-discriminant analysis (PLS-DA). It is a regression extension of PCA and its principle comprises of the separation of deductive given classes of objects.[19] This technique was conducted to develop "a model" that can be used to verify the geographical provenance of *P. major*. This was carried out on the total 35 chromatograms. These chromatograms were divided into training set and validation set. The former set consisted of four chromatograms from each location which were randomly selected. The subsequent set comprised of the remaining a chromatogram from each location. PCA and PLS-DA were carried out using Unscrambler® 9.8 from CAMO AS (Trondheim, Norway).

Statistical analysis

All values were presented as mean ± SEM or mean ± SD, $n = 3$. For multiple variables comparison, data were analyzed by ANOVA followed by Tukey test using GraphPad Prism statistical software (GraphPad Software Inc. Windows Version 5.01). Differences were considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Antioxidant activity of extracts

Radical scavenging activity of the *P. major* leaves extracts was screened against DPPH radicals. The IC_{50} values were compared for the seven extracts tested (Table 2). Samples from Solok Selatan and Agam showed the highest scavenging activity ($P < 0.05$), with IC_{50} values of 133.37 and 160.80 µg/ml, respectively. Three other samples, that is, those from Lumajang, Trawas, and Surabaya showed the next highest activity. The samples with the weakest scavenging potency were from Makassar and Lampung. Therefore, the scavenging activity of the samples in decreasing order was Solok selatan > Agam > Surabaya > Trawas > Lumajang > Lampung > Makassar. Using DPPH method, Beara *et al.* showed that IC_{50} value of *P. major* extract was 5.35 µg/ml.[14] The differences with the finding of this current study could be caused by various factors such as origin of the plant material as well as the type of extract. Previous study was carried out on aqueous methanol extract purified with petroleum ether whereas this current report has been conducted on total methanol extract. It indicated that phytochemicals constituent responsible for antioxidant activity is polar compounds.

Total phenolic and flavonoid compounds in all samples were determined by the Folin-Ciocalteu assay and the aluminum chloride colorimetric method, respectively. Serial dilution of gallic acid ($r^2 = 0.993$) and quercetin ($r^2 = 0.957$) were used as standards to set the calibration curves, which were used to calculate the total phenolic and flavonoid contents for each extract. Table 3 presents total phenolic content as well as total flavonoid compounds calculated for each extract. When the phenolic and flavonoid contents of each extract were compared with the others, it could be observed that *P. major* leaves from Agam (L4) was the highest.

Table 2: IC_{50} value of DPPH scavenging activity by extracts of *P. major* leaves.

Origin of leaves sample	IC_{50} (µg/ml)
Lumajang	263.51 ± 2.18[a]
Makassar	865.05 ± 7.68[b]
Trawas	237.24 ± 3.41[c]
Agam	160.80 ± 6.08[d]
Solok Selatan	133.37 ± 2.53[e]
Lampung	506.07 ± 2.50[f]
Surabaya	228.32 ± 8.92[c]

Results are presented as mean ± SD of IC_{50} (inhibitory concentration 50, $n = 3$). Means within column with different letters differ significantly ($P < 0.05$).

Table 3: Total phenolics and flavonoids contents of methanol extracts of *P. major* leaves.

Origin of leaves sample	Total phenolics content (g of GAE/100 g of extract)	Total flavonoids content (g of QE/100 g of extract)
Lumajang	148.84 ± 6.81[a, c]	19.61 ± 0.42[a]
Makassar	134.60 ± 5.01[a]	15.91 ± 0.81[b]
Trawas	154.38 ± 6.50[a, c]	5.12 ± 0.79[c]
Agam	210.35 ± 11.13[b]	20.57 ± 0.41[a]
Solok Selatan	198.21 ± 10.61[b]	4.61 ± 1.29[c]
Lampung	145.52 ± 1.72[a]	8.72 ± 0.20[d]
Surabaya	166.67 ±5.65[c]	8.68 ± 0.99[d]

Values are means ± SD of three determinations. Means within each column with different letters differ significantly ($P < 0.05$).

HPTLC fingerprints

Plantago major leaves from seven locations showed quite similar macroscopic features, thus caused the difficulty in samples discrimination. Moreover, as discussed in the previous section, the presence of oleanolic acid in leaves part could not be detected. In this case, marker approach could not be used to evaluate the identity and quality of *P. major* leaves. Fingerprints approach seemed to be more appropriate than marker approach. To provide informatory and accurate fingerprints, HPTLC conditions were optimized. Figure 1 presents the typical chromatogram-densitogram of *P. major* leaves samples. Eleven up to fifteen peaks were identified in each sample and all existing peaks were considered in the data analysis. All these peaks were then presented in a data matrix (data not shown), 35 x 15. Thirty-five total samples from 7 locations constituted the rows, whereas peak height of each compound appointed the columns.

Chemometrics analysis on HPTLC fingerprints

Principal component analysis (PCA)

PCA with full cross validation was applied to the data set of the 35 fingerprints of *P. major* from 7 origins. Analysis was conducted on the peaks height of the full fingerprints without any preprocessing. The PCA model with four principal components (PC) already explained 95% of total data variance (PC1 captured 63%, PC2 16%, PC3 9%, and PC4 7% of the variance, respectively). The score plot of the first two PC (Figure 2) clearly distinguished seven clusters of samples (L1-L7). The loading plot of the first PC showed that peaks number 10 and 12 are the most important peaks for clustering of samples (Figure 3).

Partial least squares-discrimination analysis (PLS-DA)

After creating the PCA model to test the presence of clusters, the next step was to build a classification model using algorithm PLS-DA. In this technique, data were analyzed to build a linear discrimination model. The signals (15 peaks height) were used as X variables, whereas the Y

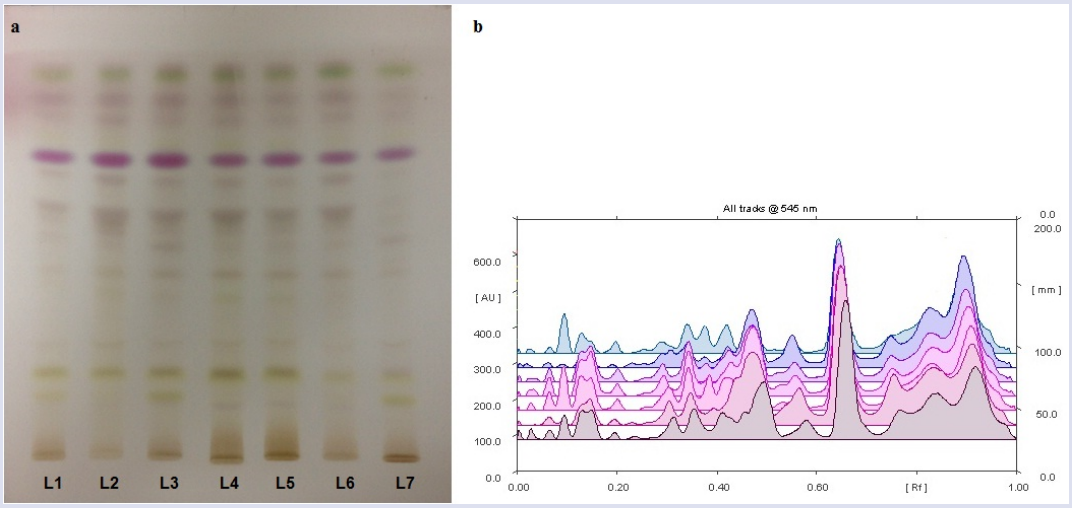

Figure 1: HPTLC-fingerprints of *P. major* leaves (a) and their densitograms (b). L1, L2, L3, L4, L5, L6, and L7 are leaves samples from Lumajang, Makassar, Trawas, Agam, Solok Selatan, Lampung, and Surabaya, respectively.

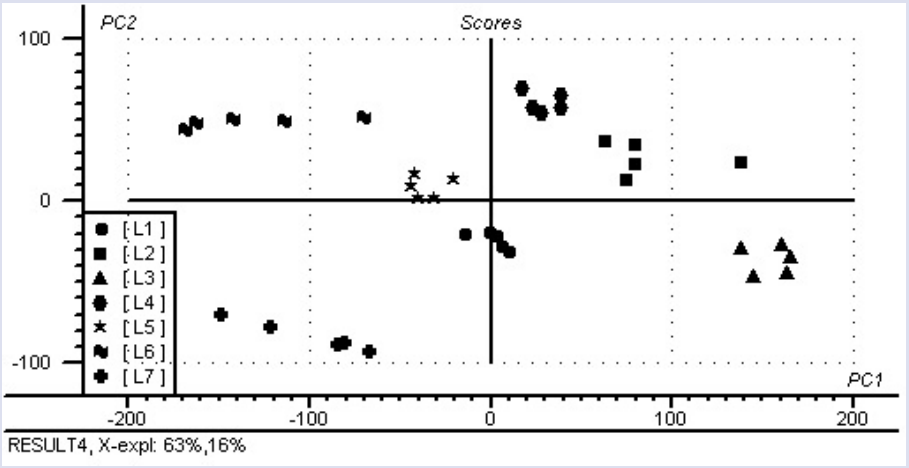

Figure 2: PCA score plot of *P. major* from different origins on the first two PCs (PC1 *vs*. PC2).

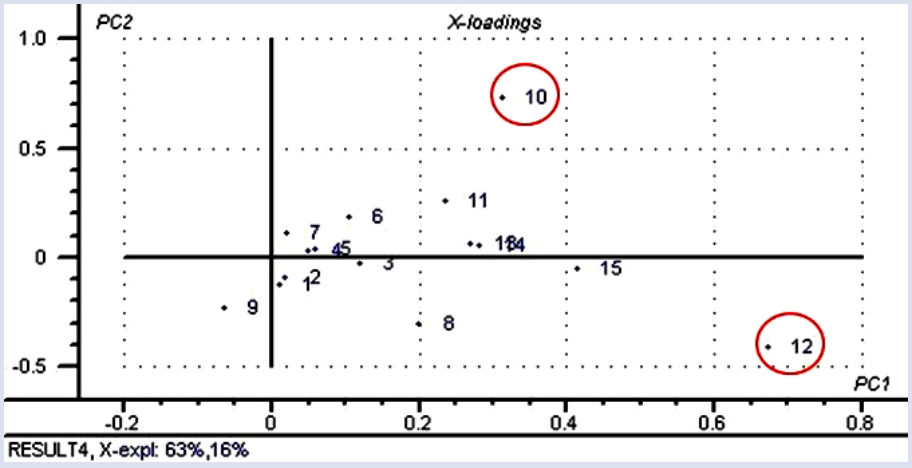

Figure 3: Loading plot from PCA for 35 samples; possible markers are with a circle.

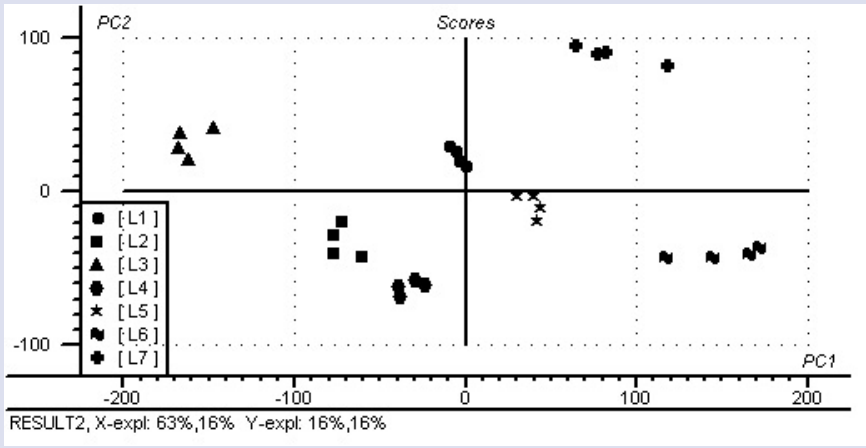

Figure 4: PLS-DA score plot of *P. major* from different origins on the first two PCs (PC1 *vs*. PC2).

Table 4: Result of classification of the *P. major* leaves samples for the prediction of samples not used in calibration (external validation data set).

Sample	Classes													
	L1		L2		L3		L4		L5		L6		L7	
	L1-prd	L1-ref	L2-pred	L2-ref	L3-pred	L3-ref	L4-pred	L4-ref	L5-pred	L5-ref	L6-pred	L6-ref	L7-pred	L7-ref
L1-v	**0.974**	1	-0.012	0	0.026	0	0.089	0	0.013	0	-0.080	0	-0.009	0
L2-v	0.032	0	**0.973**	1	-0.028	0	-0.115	0	0.059	0	-0.064	0	0.142	0
L3-v	0.038	0	0.038	0	**0.876**	1	-0.062	0	0.064	0	0.035	0	0.042	0
L4-v	0.088	0	-0.019	0	-0.029	0	**0.985**	1	-0.094	0	0.093	0	-0.024	0
L5-v	0.028	0	-0.024	0	0.049	0	0.017	0	**0.975**	1	0.020	0	-0.065	0
L6-v	-0.046	0	0.018	0	0.016	0	0.060	0	-0.050	0	**0.98**	1	0.022	0
L7-v	0.020	0	-0.026	0	0.009	0	-0.023	0	0.043	0	-0.016	0	**0.993**	1

L1, L2, L3, L4, L5, L6, and L7 represent leaves samples from Lumajang, Makassar, Trawas, Agam, Solok Selatan, Lampung, and Surabaya, respectively; -v means sample from related class used for validation set. Pred.: predicted; Ref.: reference.

variables were associated with the seven sample classes (one different Y variable for each leaves sample, with 1 or 0 depending on whether it belongs or not to the considered data group). The model obtained in this study was able to discriminate among the seven samples, as it can be seen from the PLS-DA score plot in Figure 4, where now the clusters are better distinguished than PCA cluster.

Statistical parameters of the results were obtained by PLS-DA model using the 28 calibration samples. High correlation between measured and predicted classes (R^2 in calibration is 0.9826) and low prediction errors (RMSE in calibration is 0.04) were obtained. The defined PLS-DA model was applied to classify 7 leaves samples (1 from each class) of the external validation subset. According to PLS-DA rules, a sample was considered belonging to a class when a predicted value of y was comprised between 0.5 and 1.5 for that class. Table 4 shows that for the validation samples, a 100% correct classification was achieved. The predicted values by the PLS-DA model were always very close to 1 (0.876-0.993). These results confirmed that the predictive ability of the developed classification model was very good. Therefore, it was concluded that combination of HPTLC fingerprints with chemometrics techniques, PCA and PLS-DA, has demonstrated a great potential in the discrimination and classification of herbal materials.

CONCLUSIONS

Plantago major collected from different origins in Indonesia revealed different radical scavenging activity and concentration of total phenolics as well as total flavonoids. Moreover, HPTLC fingerprint combined with the chemometrics (PCA and PLS-DA) was able to discriminate among the leaves of *Plantago major* originated from various locations. Chemical compounds represented by peaks number 10 and 12 on the HPTLC fingerprint are considered as the most important compounds for clustering of *Plantago major*.

Development of analytical methods for geographical origins of *P. major* will have positive implications for the quality control of herbal materials which will ultimately guarantee the safety and efficacy of the product.

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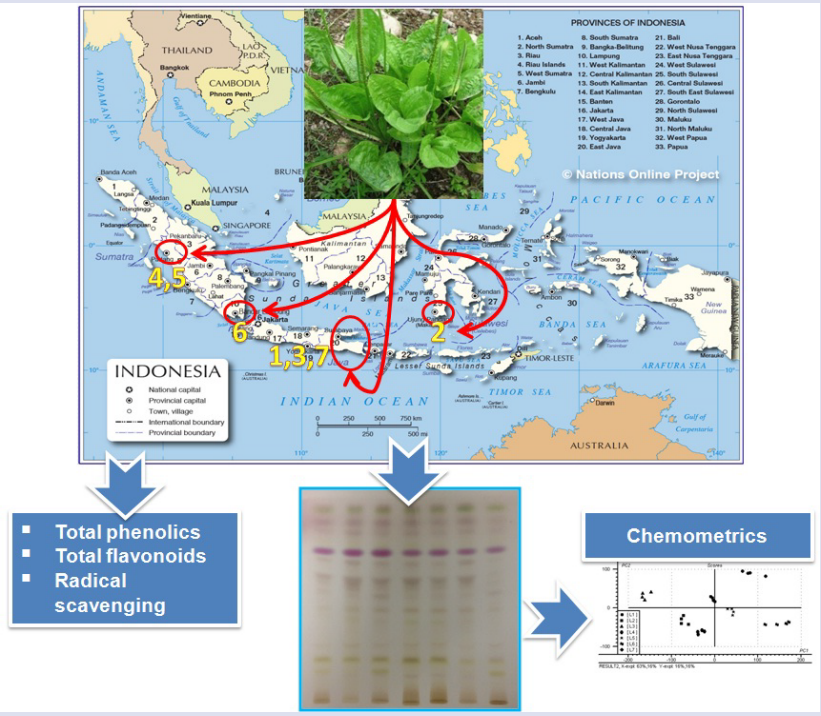

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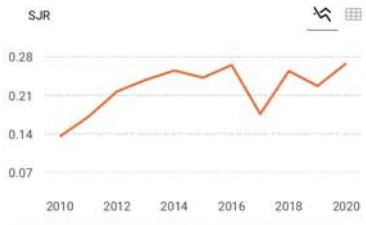

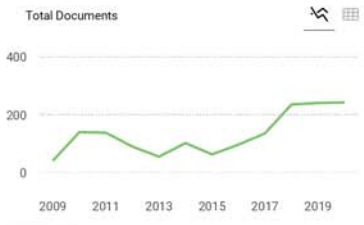

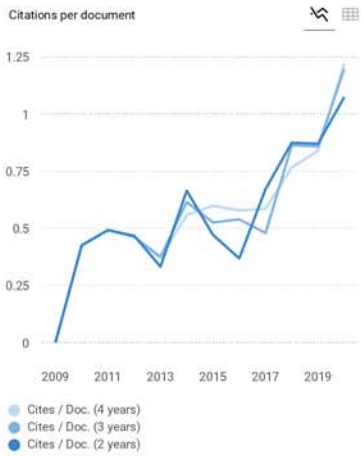

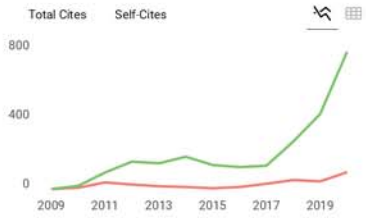

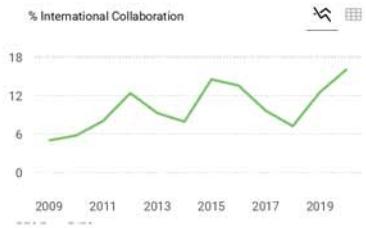

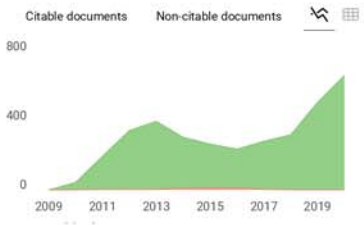

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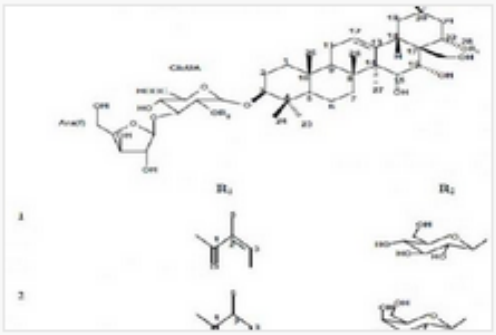

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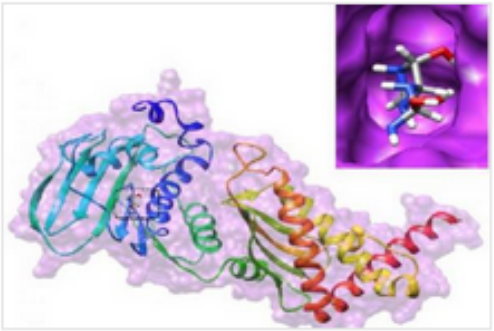

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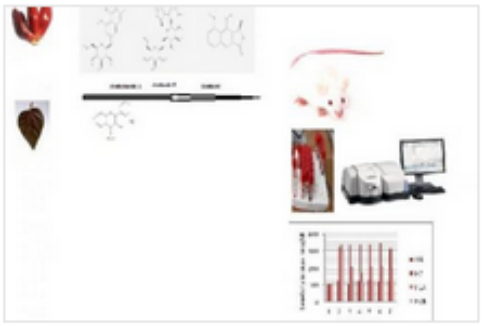

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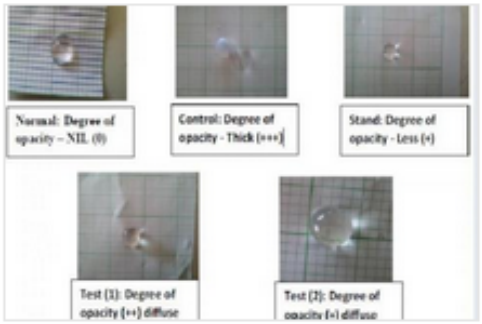

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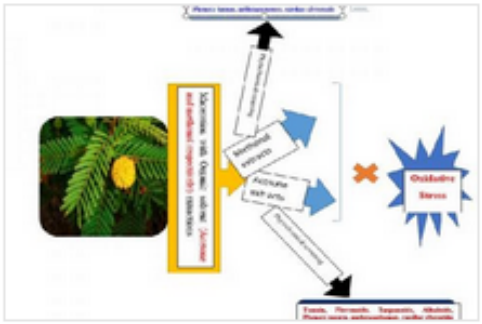

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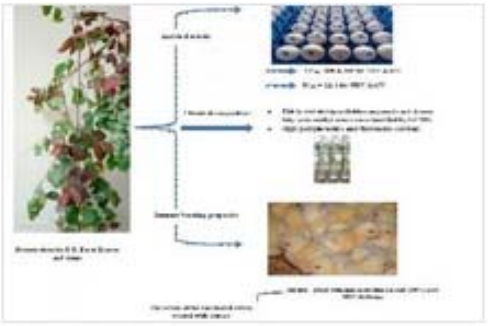

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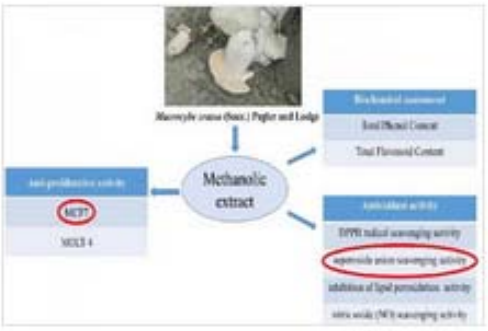

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