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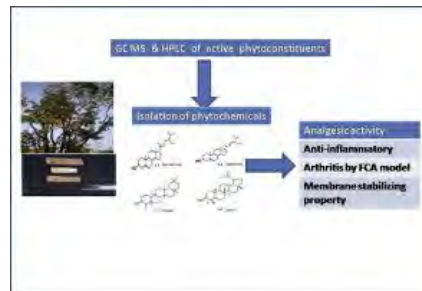
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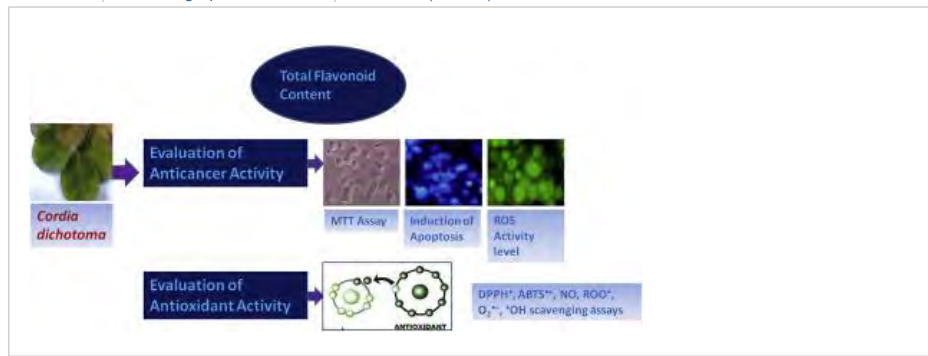
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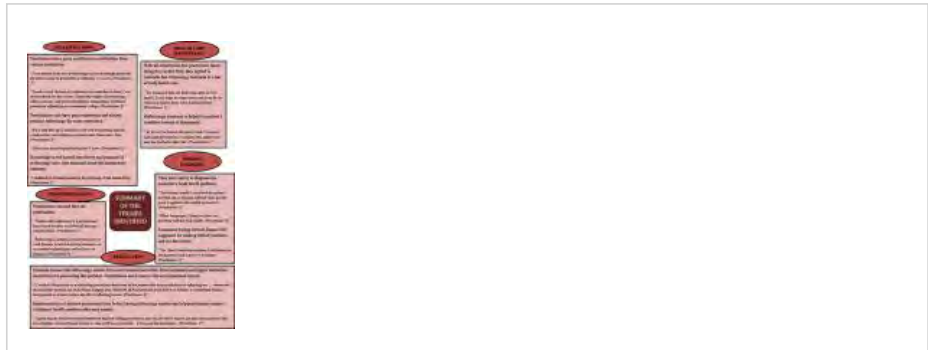
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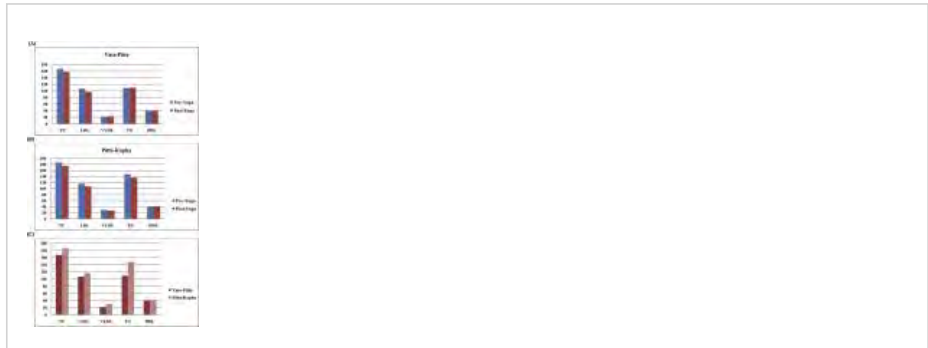
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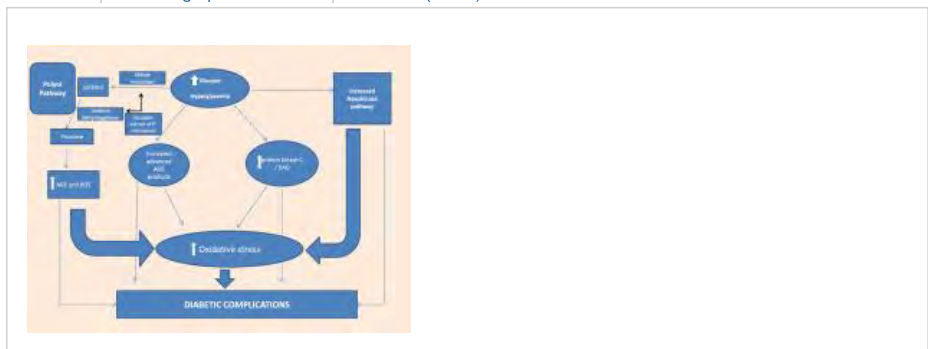
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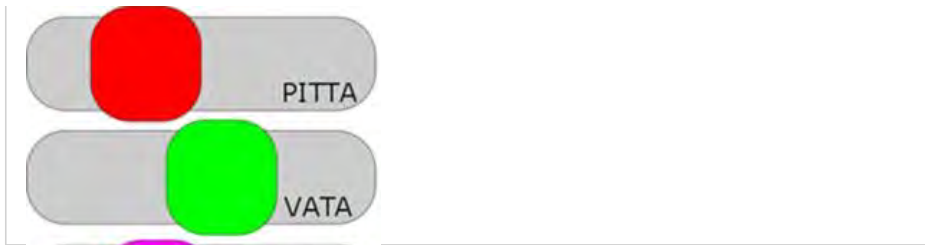
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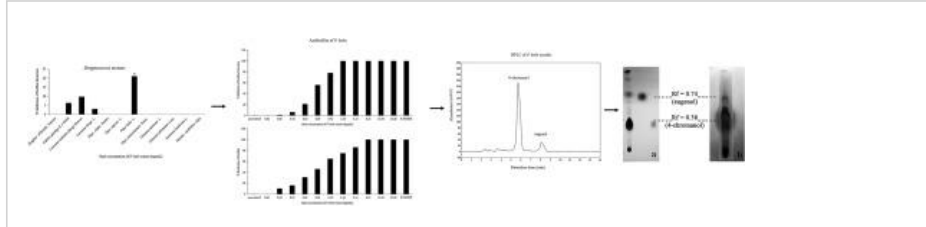


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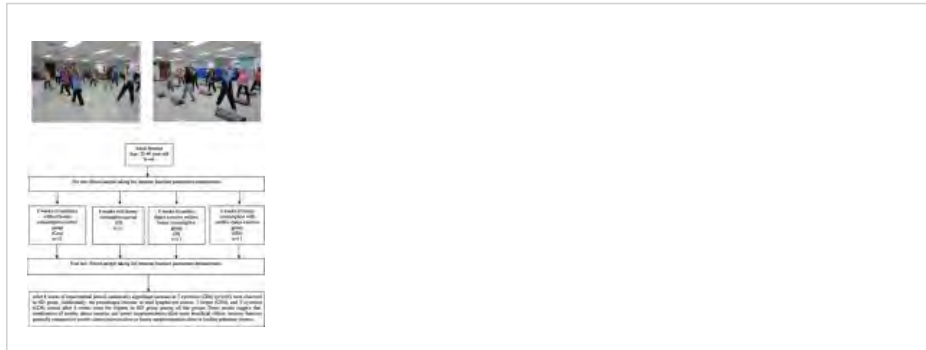


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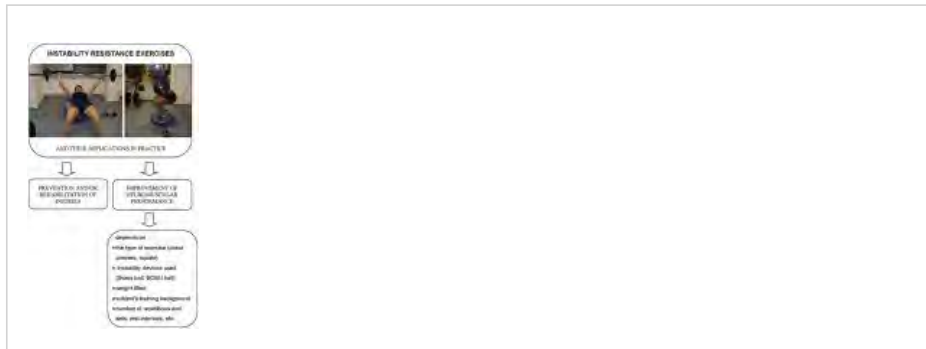


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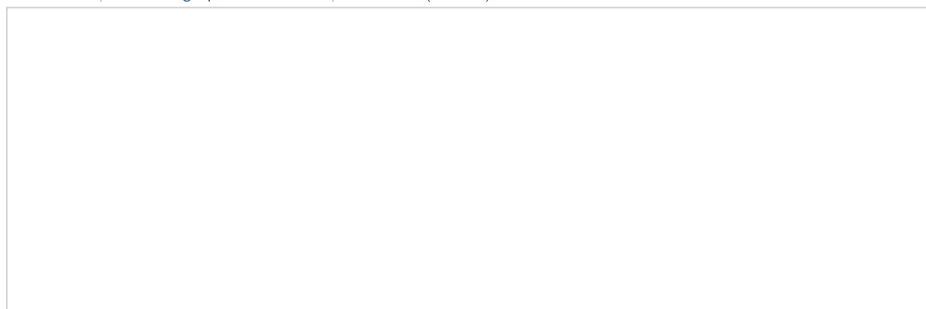


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# Authentication of Piper betle L. folium and quantification of their

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## Original Article

Authentication of *Piper betle* L. folium and quantification of their antifungal-activity

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## ABSTRACT

The TLC profiles of intra- and inter-day precision for *Piper betle* L. (PBL) folium methanol extract was studied for their peak marker recognition and identification. The Numerical chromatographic parameters (NCPs) of the peak markers, the hierarchical clustering analysis (HCA) and the principal component analysis (PCA) were applied to authenticate the PBL folium extract from other *Piper* species folium extract and to ensure the antifungal activity quality of the PBL essential oil. The spotted extract was developed with the mobile phase of toluene: ethyl acetate; 93:7, (v/v). The eluted plate was viewed with the TLC-Visualizer, scanned under absorption and fluorescent mode detection, and on each sample the *in-situ* UV spectra were recorded between 190 to 400 nm.

The NCPs profiles of intra- and inter-day precision results offered multi-dimensional chromatogram fingerprints for better marker peak pattern recognition and identification. Using the *r*-value fingerprints data series generated with this method allowed more precise discrimination the PBL from other *Piper* species compared to the marker peak area fingerprint method. The cosine pair comparison was a simple method for authentication of two different fingerprints. The ward linkage clustering and the pair cross-correlation comparison were better chemometric methods to determine the consistency peak area ratio between fingerprints. The first component PCA-loading values of peak marker area fingerprints were correlated linearly to both the bio-marker concentration as well as the antifungal activity. This relationship could be used to control the quality and pharmacological potency. This simple method was developed for the authentication and quantification of herbal medicine.

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## 1. Introduction

Authentication is a quality assessment to identify the characteristic constituents of herbal medicines.<sup>1</sup> This method is used to discriminate between authentic and counterfeit herbal medication. Through authentication methods we can assess the respective

chemical ratios of these herbal medications.<sup>2</sup> Authentication of the quantities and their chemical constituents is also important for showing the herbal medication's dosage and efficacy for consistent production. These chemical components vary depending on harvest season, geographical factors, habitat, post-harvest processing, and many other factors. These factors are elucidated by using chromatography-based authentication methods. The chromatographic profile is determined by chromatographic separating power, mode detection of the instrument, and a variety of chemical contents. These variables introduce complex chromatographic profiles. The chemometric pattern recognition method helps to identify the similarities and regularities of the chromatographic

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The modern TLC instrumentations provide the TLC-images, TLC-densitograms, which are scanned on several different wavelengths, as well as an in-situ UV-Vis spectra of each peak. This information provides multi-dimensional TLC fingerprints with accurate and precise NCPs, such as Rf-value, peak areas, and the in-situ UV-Vis spectrum of each peak.<sup>3</sup> The similarity level among chromatographic profiles of herbal medicine was calculated by using multivariate statistical methods, such as PCA and HCA. For this method, the NCP's data were transformed into Rf-peak areas data matrix. These array data points are commonly used for similarity or authentication analysis of herbal medicine. The similarity between two series of Rf-peak area chromatograms data could also be calculated by comparing both chromatograms by using the cosine correlation function ( $C_{\text{cosine}}$ )<sup>4</sup> order and the cross-correlation function ( $C_{\text{corre}}$ ).<sup>5</sup> However, the clustering result and similarity level of one cluster chromatogram may be influenced by the clustering method.

The Rf-value and in-situ UV-Vis spectrum of each peak on a chromatogram are the NCPs, which were used for identification of unknown substances.<sup>6</sup> The peak with the same Rf-value and the same UV-Vis spectrum between unknown and reference could be identified as the same compound. If a herbal medication has the two-way chromatographic fingerprint, which contains spectra and chromatograms information, this two-way chromatogram fingerprint could be settled into a reference fingerprint. The comparison of in-situ UV-Vis spectrum with a known reference by use of cross-correlation function ( $C_{\text{corre}}$ ) will deliver an *r*-value fingerprint data series. This information could be utilized for better authentication assessment of herbal medicine.

The PBL traditionally has been used for preventing body odor, and for curing diarrhea, sore throat, skin allergies, flour albus, inflammation, infection of respiratory tract, cough.<sup>6</sup> The pharmacological activity of PBL were antioxidant, antibacterial, antifungal, antileishmanial, radioprotective, antiulcerogenic, antiplatelet, immunomodulatory, mosquito larvicidal, tyrosinase inhibition, antifilarial, anti-inflammatory, anti-amoebic, and anti-giardial.<sup>6</sup> Chemical- or bio-marker identification play an important role on the herbal medicine quality control. Indonesian Herbal Pharmacopeia has described a TLC qualitative identification of PBL folium extract. This TLC system has been used to obtain the chromatogram fingerprint of PBL folium and other *Piper sp.* folium. In this study, we report the influence of difference clustering methods on HCA results and we compared the use of TLC marker peak area fingerprints and the *r*-value with the help of multivariate analysis and a paired comparison for better authentication. The antifungal activity of PBL essential oil samples was identified with TLC bioautography and quantified with agar media diffusion. The TLC fingerprints of this essential oil were assessed via PCA and the relationship between the PC1-loading plot values and their antifungal activity<sup>5</sup> was studied to develop a simple method for quantification. The aim of this study was to develop a better assessment process for better authentication analysis as well as to provide a simple multivariate analysis of the fingerprint to control the herbal medicine quality.

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## 2. Experimental

### 2.1. Chemicals and materials

All chemicals used in this study were analytical grade. The solvents were methanol (Malincord-China), toluene, ethyl acetate, TLC si GF 254 (Merck-Germany). The *Piper betle L.* folium and other *Piper* species were collected from Bali and the Java Islands.

The equipment used in the study included glassware (IWAKI-Pyrex-Indonesian), twin trough chamber 20 × 10 cm (Camag-Switzerland), semiautomatic and automatic TLC sampler (Camag-

Switzerland), TLC Scanner 4 and winCATS software (Camag-Switzerland), TLC-Visualizer (Camag-Switzerland), analytical balance (AND-Japan), moisture analyzer balance (Shimadzu-Japan), Sonication Bath (Branson-England).

### 2.2. Sample preparation

#### 2.2.1. Peak pattern recognition and precision

The dried PBL folium was grinded in a blender. The 200 mg of grinded leaves were extracted with 2 mL methanol in an ultrasonic bath for 15 min and this extraction was done in 6 times for each sample. The six replication extracts were spotted on a TLC plate (20 × 10 cm) 11 and at 5, 10, and 15  $\mu$ L. The series were spotted on one plate for intra-day precision and two different plates on 3 various days for inter-day precision. The series placed samples were developed with mobile phase (toluene: ethyl acetate; 93:7, v/v) in a twin-trough chamber. The developed spots were viewed under TLC-visualizer and their image was captured under UV lamp at wavelengths of 254 nm and 366 nm and under white R lamp, scanned under UV light ( $\lambda = 210, 283, \text{nm}$  absorption mode and 366 fluorescence mode), and the in-situ spectra of each peak were recorded.

#### 2.2.2. Authentication

The 200 mg grinded PBL folium was collected from 21 different regions and 5 other species of *Piperaceae* (*Piper albi* and *Piper nigrum*, *Piper retrofractum*, *Piper cubeba*, and *Piper oratum*). They were extracted with 2 mL methanol in an ultrasonic bath for 15 min, and 10  $\mu$ L of those extracts were spotted on a plate. The TLC developing, TLC image viewer, the densitograms scanning, and the in-situ UV spectrum recording were done as well as on previous method.

#### 2.2.3. Biomarker determination

The 2 kg fresh chopped *P. betle L.* folium collected from 9 different regions were placed in water and then distilled. The distilled water was saturated with NaCl and the essential oil collected into dark brown vials. The residual water was dried with NaSO<sub>4</sub> anhydrate. The 4  $\mu$ L essential oil was diluted with 1 mL ethanol and each TLC plate was given 30  $\mu$ L of the prepared solution. Spotted series were completed on five replication. The developing plate, TLC image viewer and the in-situ UV spectrum recording were done like previous method. The densitograms detection was scanned under 283 nm. Each track was cut into 1 × 10 cm sections. Afterward, plates were sterilized by UV-lamp in clean air bench for 20 min.<sup>7</sup> The 75  $\mu$ L suspension of *Candida albicans* ATCC 10231 in a concentration of  $1.5 \times 10^8$  CFU/mL was diluted in 15 1 of soboraud dextrose agar (SDA), then was poured into the petri dish and allowed to solidify. The sterilized plate was faced down to agar medium, allowed to pre-diffuse for one hour and then incubated at 37 °C for 24 h.<sup>8</sup> Inhibition zones around TLC spots were noted. These studies were done for each essential oil of *P. betle L.* folium collected from 9 different regions.

#### 2.2.4. Antifungal activity

The 15 mL SDA was poured into the sterile petri dish and allowed to solidify. The 19 *C. albicans* inoculum suspension was swabbed uniformly with sterile cotton swabs and allowed to stand for 15 min. The 15  $\mu$ L of diluted essential oil (100  $\mu$ L/mL) was loaded on 6 mm sterilized filter paper discs and placed on the surface of a medium. This petri dish was incubated at 37 °C for 24 h.<sup>9</sup> Afterward, the inhibition zones (IZ) were measured with a ruler. The antifungal activity of each essential oil of *P. betle L.* folium collected from 9 different regions were performed in triplicate.

### 2.3. TLC-densitometry

The TLC 20 cm × 10 cm aluminum-backed plates were pre-coated with a 0.1 mm layer of silica gel 60 F254 (Merck, Darmstadt, Germany), prewashed with methanol and activated (120 °C for 30 min). The dried plates were equilibrated and stored at room temperature. Samples and standard solutions of different concentrations were applied to the plates in a band form in length of 3.5 mm, for the first application x = 20 mm, y = 10 mm, and 6.5 mm space between tracks by use of an automatic sampler (ATS) applicator fitted with 25 µL syringe and N<sub>2</sub> gas.

The twin-trough chamber (20 × 10 cm) was saturated with mobile phase vapor for 30 min, the spotted samples were developed with mobile phase to a distance of 9 cm at room temperature, and the developed plates were dried at 60 °C for 5 min.

The dried plates were scanned under UV light, with slit length of 80% of the total width of the spots and the noise factor value was 2.6, with a scanning speed of 20 mm s<sup>-1</sup>, and a data resolution of 100 µm/step. The *in-situ* UV spectra detection of each peak were recorded in all assigned peaks. The parameters integration peak was set on 14: r factor: Savit-Sky-Golay 19, lowest slope, peak thresholds (minimum slope: 5, minimum height: 10 AU, minimum area: 50 AU).

### 2.4. Data processing

The cross-correlation function has been used to compare the two measured *in-situ* UV spectra of both peaks, and this comparison resulted in a correlation value (*r*-value). The similarity levels between a pair chromatogram fingerprints were calculated with the cosine correlation value (*C*<sub>cosine</sub>) and correlation value (*C*<sub>correl</sub>). The two chromatogram fingerprints could see as two vectors *a* and *b* and the *C*<sub>cosine</sub> and the *C*<sub>correl</sub> equations are described by (1) and (2):

$$C_{\text{cosine}} = 100 \frac{(NCP_{a1} \cdot NCP_{b1} + NCP_{a2} \cdot NCP_{b2} + \dots + NCP_{at} \cdot NCP_{bt})^2}{(NCP_{a1}^2 + \dots + NCP_{at}^2)(NCP_{b1}^2 + \dots + NCP_{bt}^2)} \quad (1)$$

$$C_{\text{correl}} = 100 \frac{\sum_{t=1}^n (NCP_{at} - \overline{NCP_{at}})(NCP_{bt} - \overline{NCP_{bt}})}{\sqrt{\sum_{t=1}^n (NCP_{at} - \overline{NCP_{at}})^2 \sum_{t=1}^n (NCP_{bt} - \overline{NCP_{bt}})^2}} \quad (2)$$

where, the vector *a* is constructed with *t* observations (NCP<sub>a1</sub>, NCP<sub>a2</sub>, ..., NCP<sub>at</sub>) and the vector *b* is NCP<sub>b1</sub>, NCP<sub>b2</sub>, ..., NCP<sub>bt</sub>, respectively. The observed TLC-NCPs of variable chromatograms fingerprints (vectors), with the help of software, were arranged into the rectangular matrix (*t* observations × *n* variables). The PCA and HCA calculation of matrix data sets were done with the help of Minitab 17 statistical software. The PCA calculation was done on a covariance type matrix.

## 3. Results and discussion

### 3.1. Marker peaks recognition and identification

The densitograms of intra- and inter-day precision test, which was scanned under absorption mode on 210 nm presented in Fig. 1. A few peaks were detected in several tracks with the broad range of hRF-value and their appeared randomly. These peaks possibly were the impurities. They possessed different *in-situ* UV spectra. These peaks, which were detected in all tracks either on intra- or inter-

day plates, had close Rf-value and presented relatively close *in-situ* UV spectrum. These are assumed to be the marker peaks.

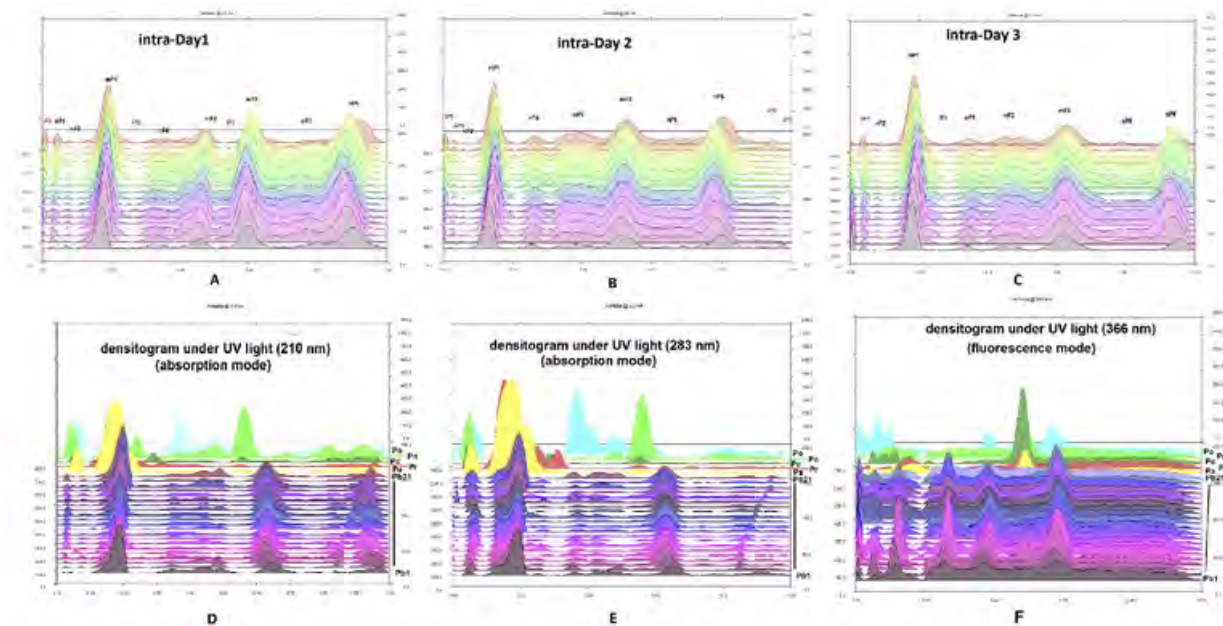
The Indonesian herbal pharmacopeia appointed eugenol as a chemical marker of PBL. The eugenol was detected on hRF-value of 54.1. Comparison the spectra between sample peak with the same hRF-value eugenol peak obtained the *r*-value of 0.947 (see Fig. 2). This peak could be signed as eugenol and named as mP3. The mP3 peak was also detected in all chromatogram tracks of extract, and it showed close hRF-value either on intra-day or inter-day precision. The spectrum of each peak was compared, and the *r*-values were more than 0.900. This result could be used to identify a marker peak of chromatogram profile PBL folium extract. Based on this result, 11 assumed marker peaks were found such as nP1, nP2, nP3, nP4, nP5, nP6, mP1, mP2, mP3, fP1, and fP2 (see Fig. 3). The mP1 and mP2 marker peaks also presented relatively closer *in-situ* UV spectrum with eugenol, the *r*-values of mP1 were more than 0.8889 and the *r*-values of mP2 more than 0.8088, respectively. These possibly indicate that they have closer chemical structure with eugenol.

The mean peak areas, the %RSD and regression parameters of 11 assumed markers, which scanned on the different wavelength and mode presented in Table 1. The all considered marker peaks areas showed good correlation with their spotted volumes (*r*-reg > 0.9), except nP1-210, nP2-210, and nP3-283. The peak nP1 was just detected under 210 nm, and presented *r*-reg of 0.777 and this marker peak was minor. The nP2 presented good correlation regression when scanned under 283 nm. The nP3 peak fluorescence under 366 nm and their scanned peaks areas under 366 nm fluorescence mode was linear correlate to their spotted volumes. Each presumable marker peaks should possess close hRF value or identical *in-situ* UV spectra and the peak should be detected in all chromatogram tracks both intra- and inter-day precision. The peak marker areas should be linearly correlated to their concentration levels. These results have been used to recognize and identify the marker peak of the phytochemical thin layer chromatographic profiles.

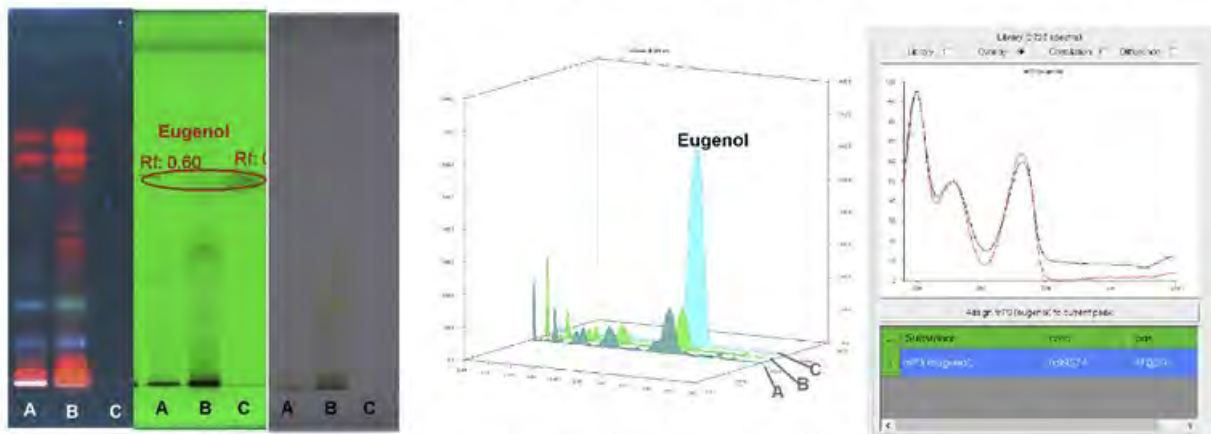
### 3.2. Authentication of TLC fingerprint of methanol PBL folium extract

The fingerprint data series, which were constructed by intra- and inter-day precision markers peak areas, were used to calculate the similarity level of the same samples. The PCA eigenvalues recommended all inter-day fingerprints in one grouping, with similarity-level of 95.1%. The HCA calculation through various linkage methods of intra-day fingerprints data series delivered the similarity level between 82.7–99.5% and between 40.9–98.1% for the inter-day data series. The ward linkage method resulted in the lowest similarity values (82.7–93.4 for the intra-day data set and 40.9% for inter-day data series). The single linkage method introduced the highest similarity values among other linkage methods (98.1–99.5 for intra- and inter-day data series). The pair comparison methods delivered different similarity value ranges of individual fingerprint comparisons. The *C*<sub>correl</sub> arranged between 79.7 to 99.9%, but the *C*<sub>cosine</sub> assigned 81.0–99.9%. The various linkage methods or particular comparison methods delivered different similarity levels of the intra- and inter-day fingerprints data series. This could potentially confuse authentication decisions, although all fingerprint data series were from the same sample, These fingerprints should present high similarity level.

The wide variation ward similarity values between intra- and inter-day fingerprints data series could be contributed by marker peak area variation between different spotted volumes. The score plot is used to understand relationships between marker peaks areas (observations data). The PC1-score value of each marker correlated linearly to their peak areas, with *r*-reg > 0.92 (see Fig. 4a



**Fig. 1.** Intra- and inter-day precision densitograms (A–C) and the densitograms of methanol PBL extracts from different geographic location and other *Piper* species under difference scanned modes (D–F). The Pb: *P. betle* L. from 21 different harvesting areas, Pa: *Piper albi*, Pn: *Piper nigrum*, Pc: *Piper cubeba*, Pr: *Piper retrofractum* and Po: *Piper ornatum*.



**Fig. 2.** Identification of eugenol on PBL folium extract. A and B were spotted extract PBL folium and C was the eugenol reference.

and b). The inter-day precision presented higher %RSD-value than the intra-day precision (see Fig. 4c). The great variation marker peak areas on inter-day data series reduced ward similarity, but these %RSD changes influenced not significantly to the single similarity.

The authentication method should be developed to differentiate original medicines from counterfeit medications and also will help to evaluate the stability of their constituents bio-markers quantities to ensure their efficacy and repeatability.<sup>2</sup> The biomarker stability components are reflected by the consistency of the ratio between bio-marker peak areas. Table 2 described the influence of marker peak area ratios on the PCA and HCA calculation results. The relative constant of marker peak area ratio between fingerprints (Lv:1,2,3, see Table 2) governed the close PC1 score values and these fingerprints possessed the similarity near 100%. The marker area

ratio changing was done through the fixing of one markers area (Lv(mP3):1,2,3; Lv(mP2):1,2,3; Lv(mP1):1,2,3, see Table 2) on the three different spotted volumes. These changed ratios reduced their similarity level. The HCA ward linkage method and the  $C_{\text{correl}}$  pair comparison detected these ratio changing sensitively in compare to other HCA linkage methods as well as  $C_{\text{cosine}}$  pair comparison. This simulation presented the similarity level near to 100% indicated, that the entire fingerprints possessed the relative close marker peak area ratio. Variation of the marker peak area ratio will decrease their similarity level.

The densitograms TLC images of 21 differences PBL samples, harvested from different geographic locations, and five other species of *Piper* (*P. albi*, *P. nigrum*, *P. cubeba*, *P. retrofractum* and *P. ornatum*) are showed in Fig. 1. The PCA eigenvalue was recommended these fingerprints data series into three groups. The

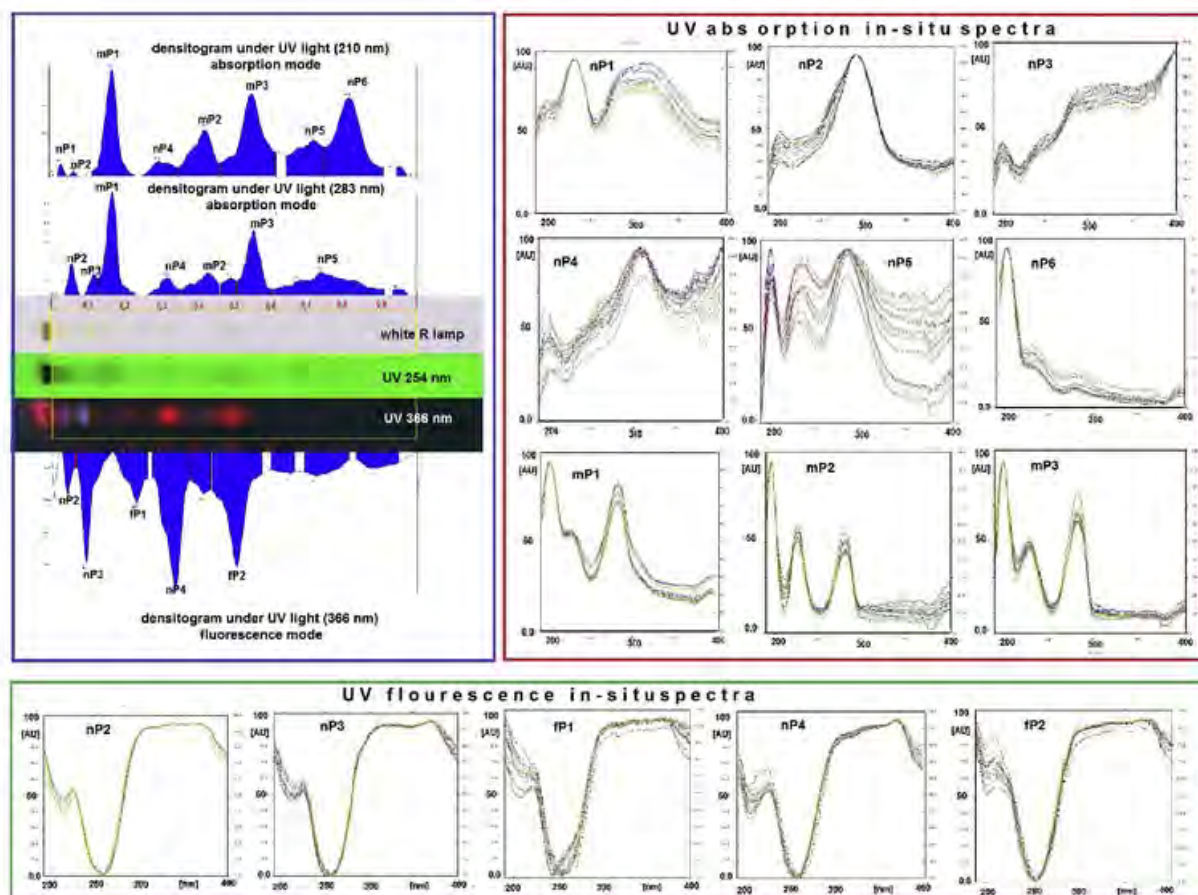


Fig. 3. The multi-dimensional TLC fingerprint of PBL folium.

**Table 1**

The inter-day precision marker peak areas on different spotted volumes and their regression parameters.

Assumed marker-scanned $\lambda$ (nm)	Baseline correction peak areas			Regression Parameters		
	Peak areas on different spotted volume [mean (%RSD)]			Reg-equ	r-reg	sdv
	5 ( $\mu$ L)	10 ( $\mu$ L)	15 ( $\mu$ L)			
nP1-210	182.5 (58.5)	351.1 (34.1)	474.2 (18.0)	$Y = 44.2 + 29.2x$	0.777	30.4
nP2-210	101.3 (27.3)	120.2 (41.3)	152.6 (21.1)	$Y = 73.3 + 5.1x$	0.518	29.4
nP2-283	1219.2 (11.7)	1896.3 (9.2)	2271.6 (5.7)	$Y = 743 + 105x$	0.941	9.1
nP2-366	1209.5 (5.2)	1540.7 (6.5)	1809.7 (6.5)	$Y = 919 + 60x$	0.941	6.2
nP3-283	289.1 (14.1)	393.6 (17.8)	434.5 (15.6)	$Y = 227 + 14x$	0.715	16.4
nP3-366	3500.1 (3.4)	5713.5 (4.1)	7660.2 (5.1)	$Y = 1464 + 416x$	0.989	4.8
nP4-210	655.9 (6.0)	1063.5 (3.7)	1402.4 (9.8)	$Y = 291 + 74x$	0.967	8.14
nP4-283	737.0 (9.9)	1367.1 (6.4)	1830.4 (6.2)	$Y = 202 + 111x$	0.979	7.8
nP4-366	4483.3 (3.6)	7963.7 (3.6)	9485.6 (7.1)	$Y = 2140 + 521x$	0.961	9.2
mP1210	5021.5 (1.3)	7529.4 (2.1)	10319.3 (6.5)	$Y = 2325 + 529x$	0.986	5.2
mP1-283	4220.5 (4.1)	6746.7 (3.5)	8340.0 (5.2)	$Y = 2316 + 411x$	0.978	5.8
mP2-210	1528.6 (5.3)	3342.6 (7.2)	5064.5 (4.3)	$Y = -195 + 349x$	0.992	5.7
mP2-283	589.3 (7.8)	808.5 (4.9)	966.5 (2.5)	$Y = 410 + 37x$	0.972	5
mP3-210	4556.3 (5.6)	6770.8 (2.0)	7769. (1.2)	$Y = 3406 + 323x$	0.973	5.4
mP3-283	3034.3 (5.5)	4395.7 (7.1)	5614.0 (2.2)	$Y = 1771 + 257x$	0.981	5.1
nP5-283	1368.5 (2.9)	2750.1 (4.7)	4482.0 (2.7)	$Y = -239 + 310x$	0.995	4.9
nP6-210	5044.7 (6.8)	7585.1 (3.3)	9882.3 (7.0)	$Y = 2727 + 472x$	0.977	6.2
IP1-366	349.4 (12.4)	1121.0 (7.5)	1703.2 (3.2)	$Y = -295 + 135x$	0.991	7.3
IP2-366	4570.1 (3.4)	6288.4 (2.5)	7990.3 (5.0)	$Y = 2794 + 351x$	0.989	3.8

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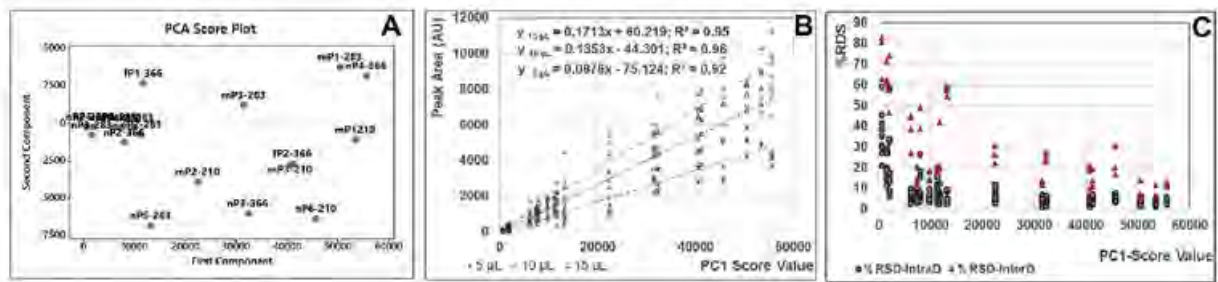


Fig. 4. The PCA score plot graph, relationship between PC1-score value of each marker and their peak area, and the %RSD distribution.

Table 2

Relation between the marker peak area ratio variation on the PCA and HCA calculation results.

Spotted volume	Simulated marker peak area ratio AUCmP3:AUCmP2:AUCmP1 (ratio)				Equation relationship between PC1-PCA-score-value and similarity level
	Lv:1,2,3	Lv(mP3):1,2,3	Lv(mP2):1,2,3	Lv(mP1):1,2,3	
5 $\mu$ L	3217:2417:4973 (1.3: 1.0: 2.1)	3217:2417:4973 (1.3: 1.0: 2.1)	3217:2417:4973 (1.3: 1.0: 2.1)	3217:2417:4973 (1.3: 1.0: 2.1)	$y = -1E - 06x^2 + 0.014x + 57.68$ , $R^2 = 0.999$
10 $\mu$ L	4954:4142:6677 (1.2: 1.0: 1.6)	3217:4142:6677 (0.8: 1.0: 1.6)	4954:2417:6677 (2.0: 1.0: 2.8)	4954:4142:4973 (1.2: 1.0: 1.2)	$y = -1E - 06x^2 + 0.016x - 52.5$ , $R^2 = 0.999$
15 $\mu$ L	6487:5364:9250 (1.2: 1.0: 1.7)	3217:5364:9250 (0.6: 1.0: 1.7)	6487:2417:9250 (2.7: 1.0: 3.8)	6487:5364:4973 (1.2: 1.0: 0.9)	$y = -1E - 06x^2 + 0.023x + 28.52$ , $R^2 = 0.999$
Cluster linkage	Similar level between Lv (%)				Equation relationship between PC1-PCA-score-value and similarity level
	Lv:1,2,3	Lv(mP3):1,2,3	Lv(mP2):1,2,3	Lv(mP1):1,2,3	
Average	99.98	90.62	97.42	45.04	$y = -1E - 06x^2 + 0.014x + 57.68$ , $R^2 = 0.999$
Centroid	99.98	90.68	97.42	48.12	$y = -1E - 06x^2 + 0.016x - 52.5$ , $R^2 = 0.999$
Complete	99.97	89.2	97.42	27.62	$y = -2E - 06x^2 + 0.023x + 28.52$ , $R^2 = 0.999$
McQuitty	99.98	90.62	97.42	45.04	$y = -1E - 06x^2 + 0.016x - 52.5$ , $R^2 = 0.999$
Median	99.98	90.68	97.42	48.12	$y = -1E - 06x^2 + 0.014x + 57.68$ , $R^2 = 0.999$
Single	99.98	92.04	97.42	62.47	$y = -8E - 07x^2 + 0.008x + 76.43$ , $R^2 = 0.999$
Ward	99.97	87.58	96.56	30.82	$y = -2E - 06x^2 + 0.019x + 43.51$ , $R^2 = 0.999$
Cosine	99.10	94.16	96.72	87.67	$y = -0.0017x + 108.96$ , $R^2 = 0.999$
Correl	99.94	87.41	94.83	-44.79	$y = -4E - 06x^2 + 0.058x - 87.91$ , $R^2 = 0.995$

all HCA linkage methods could not draw aside the *P. nigrum* fingerprint from the cluster the PBL fingerprints. The HCA complete and single linkage methods have clustered some of the PBL fingerprints outside their group. The single relation between a pair of 1-way fingerprints of PBL (Bet-Bet) were arranged between 22 to 100% for the  $C_{\text{cosine}}$  and 4–100% for the  $C_{\text{correl}}$ , respectively. The paired comparison of the 1-way PBL fingerprints versus the other *Piper* sp fingerprints (Bet-Other sp.) resulted in the similarity value between 4–84% for  $C_{\text{cosine}}$  function comparison and between -40 to 80%, respectively. Both single comparisons were obtained by the overlapping C-value. It means that these 1-way fingerprints could not differentiate PBL fingerprints from the other species of *Piperaceae*.

The 2-way fingerprint data series were constructed by the marker peak area data, where the marker peak identification was based both of their Rf-values and their spectrum correlation between fingerprint reference and sample. The PCA eigenvalue indicated that the fingerprint samples needed to be grouped into 4 clusters. The HCA discriminated the TLC fingerprints of *P. albi*, *P. cubeba*, *P. retrofactum* and *P. ornatum* from the cluster of the *P. betle* L., but not for the *P. nigrum* fingerprint. Both single comparison functions could not discriminate the PBL-TLC fingerprints from the other *Piper* species fingerprints. Authentication of herbal plants should differentiate the original species from counterfeit samples. Tests that are not influenced by marker area peak factors. However, using this peak area fingerprint method was not always successful to discriminate original species from false samples.

The *in-situ* UV spectrum of each chromatogram peak has been used for peak marker identification. The cross-correlation

comparison between both *in-situ* UV spectra of the marker reference and the identified marker resulted in the  $r$ -value. Arrangement of these  $r$ -values into a rectangular matrix was possible to analyze their cluster and similarity. Fig. 5 describes the PCA-loading plot, clustering, C-values, and distribution of  $r$ -values for PBL and other *Piper* species fingerprints data series. All HCA linkage methods successfully discriminated the PBL fingerprints from the other species of *Piperaceae*. The similarity-levels of the *P. betle* L. cluster were 80.4% for average linkage, 82.6% for centroid linkage, 64.4% for complete linkage, 80.1% for Mcquitty linkage, 84.8% for median linkage, 87.9% for single linkage and 39.7% for ward linkage, respectively. The cosine correlation function could differentiate the PBL fingerprints from other species of *Piperaceae* fingerprints. On the other hand,  $C_{\text{correl}}$  values distributed overlap between the *P. betle* L. fingerprints from the fingerprints of other species. The  $r$ -value fingerprint was independent to the peak area. The single linkage clustering method presented the highest similarity level. The cosine function was the better method to describe single relationships between a pair  $r$ -value fingerprints and could also precisely discriminate the  $r$ -value the PBL fingerprints from the non-PBL fingerprints. This method could be implemented in authentication of other herbal medicines to insure quality and authenticity of the medication.

### 3.3. Quantification

The TLC-bioautography presented two inhibition zones (13) to *C. albicans* ATCC 10231. The first zone was the peak with Rf value of 0.46 and the second was peak Rf-value of 0.62. Both peaks were



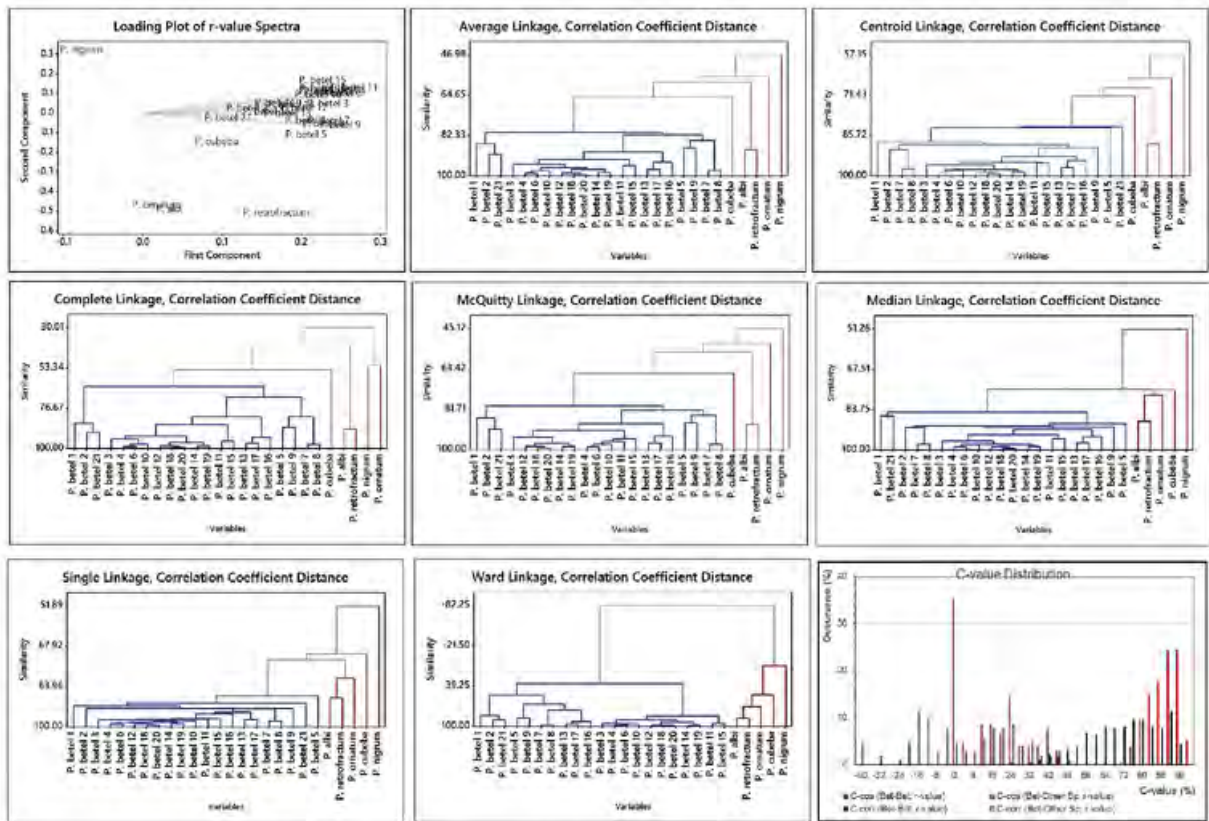


Fig. 5. PCA, HCA, and pair comparison of the *r*-values TLC-fingerprints data of PBL and other *Piper* sp.

mP2 and mP3 (see Fig. 6). The ideal quantification of herbal medicine is a determination of their bio-markers, which is responsible for the claimed pharmacological effect. Most bio-markers are not clearly defined or identified, so it is hard to find the chemical reference. Based on these difficulties, it was proposed for quality control to use the fingerprint of plant extract. This has been used for the quality bioassay control and as a fingerprint reference standard.

The multivariate analysis required a rectangular matrix with a minimum of 3 observation data points for better results. The peak height readout of a scanned spot was start-height (sh), maximum-height (mh), and end-height (eh), so each peak possessed three observation data points. The multivariate analysis of both biomarker peaks gave six observation data points for each fingerprint parameter. The *r-reg* value of correlation PC1 loading versus spotted volumes was 0.975, and the *r-reg* value of relationship PC1-loading versus total height peaks of mP2 and mP3 was 0.998 (see Fig. 7a–b). The inhibition zone (IZ) of 9 differences PBL essential oils arranged between 17.4–31.5 mm. The relationship antifungal activity (IZ) of both markers (mP2 and mP3) presented in Fig. 7c. The *r-reg* value of mP3 peak heights was 0.531, 0.926 for mP2 peak height, and 0.988 for total peak height of mP2 and mP3, respectively. The antifungal activity of PBL essential oil samples increased synergies to their contained mP2 and mP3. It means that the both markers possessed the synergies pharmacological effect to inhibit *C. albicans*. The PC1 loading plot of TLC *P. betle* L. essential oil fingerprints correlated linearly to their antifungal activity (see Fig 7d), with *r-*

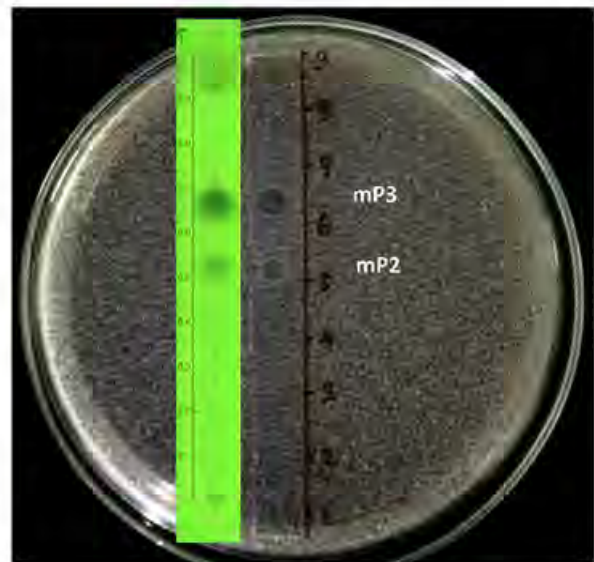


Fig. 6. TLC bioautography of PBL essential oil to the *Candida albicans* ATCC 10231.

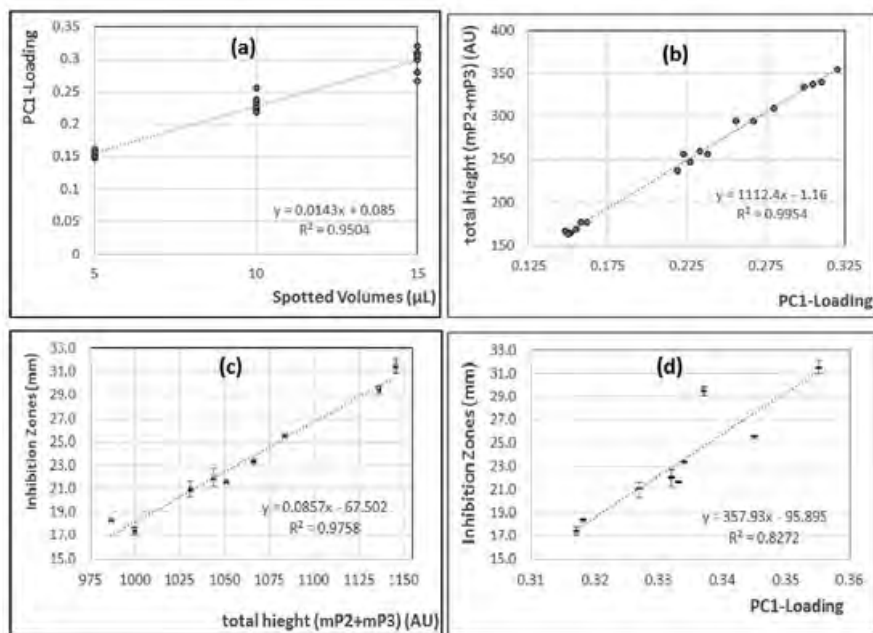


Fig. 7. The relationship between the multivariate analysis results of inter-day precision bio-marker fingerprints, their NCPs (a–b), and the antifungal activity (c–d).

reg of 0.909. The phytochemical chromatographic fingerprint consisted of multi-marker peaks areas or peak heights profile. The PCA loading plot could convert each multi NCPs fingerprint profile into a single PC1-loading plot value. These PC1-loading values were correlated linearly with their spotted volumes (concentration levels) as well as with their antifungal activity (IZ). This relationship could be used to determinate and to control the quantity bio-marker constituents.

#### 4. Conclusion

The chemometric analysis helps us to understand the underlying data structure and discriminate the variation of observed NCPs data between fingerprints. The intra- and inter-day precision study of the methanol *P. betle* L. folium extract could be used to recognize and identify their marker peaks. The *r*-value of the fingerprints were meaningful for authentication of *P. betle* L. and this fingerprint could differentiate *P. betle* L. from other plant extracts. The HCA ward similarity level varied significantly with their %RSD values as well as with their consistency marker peak areas-ratio. The PC1 loading value reflected the concentration marker level as well as the pharmacological potency. This developed method was an easy way to control the originality and to ensure the quantification of the *P. betle* L. as an antifungal herbal medicine.

#### Conflict of interest

None declared.

#### Acknowledgments

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