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Original Article

Thin Layer Chromatography Fingerprinting and Clustering of *Orthosiphon stamineus* Benth. from Different Origins

Kartini Kartini[1,*], Ervina Rustiana Dewi[1], Fandi Achmad[1], Nikmatul Ikhrom Eka Jayani[1], Mochammad Arbi Hadiyat[2], Christina Avanti[3]

ABSTRACT

Introduction: *Orthosiphon stamineus* has been widely used across Asian countries for the treatment of various diseases. The quality of herbal medicine determine its safety as well as efficacy; and geographical origin is important factor contributing on the quality of herb and its products. **Materials and Methods:** Thin Layer Chromatography (TLC) method combined with chemometric, Principal Component Analysis (PCA), has been employed to evaluate the quality of *Orthosiphon stamineus* leaves collected from eleven origins in Indonesia. **Results:** The results showed that mobile phase suitable for *Orthosiphon stamineus* was chloroform, dichloromethane, ethyl acetate (7:4:1). The method used has met the requirements of TLC system stability and precision. TLC-fingerprints analyzed with chemometrics showed an ability to discriminate *Orthosiphon stamineus* from various origins. PCA score plot of the first two principal components (PC) clearly distinguished 3 clusters of samples, whereas the loading plot of the first two PC showed that compounds with the Rf values of 0.0-0.1, 0.1-0.2, 0.2-0.3, and 0.9-1.0 are the most important compounds for clustering of samples. **Conclusions:** TLC-fingerprint combined with the PCA was able to discriminate among the leaves of *Orthosiphon stamineus* originated from various locations. TLC-fingerprints analyzed with chemometrics can be used as an alternative of marker-oriented method to evaluate the quality of *Orthosiphon stamineus*.

Key words: Geographical origin; Herbal medicine; Marker; Principal component analysis; Quality; TLC.

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INTRODUCTION

Orthosiphon stamineus (Indonesia: *Kumis kucing*) is a member of the Lamiaceae family. This plant has been empirically used in several Southeast Asian countries, such as Indonesia, Malaysia, Thailand, Vietnam, and Myanmar. *Orthosiphon stamineus* (OS) in Indonesia is traditionally used for diuretic, rheumatic, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorders, gonorrhea, syphilis, kidney stones, nephritis, gout arthritis, and antipyretics.[1] Various studies on OS and its compounds indicated that this plant has antioxidant, cytotoxic, diuretic, nephroprotective, antidiabetic, antihypertensive, anti-inflammatory, antimicrobial, anti-obesity, and hepatoprotective activities, as well as activity on the cardiovascular system.[1-5] Bioactivities of OS has been attributed by its various chemical constituents classified as monoterpenes, diterpenes, triterpenes, essential oil, sterols, saponins, flavonoids, and organic acids.[1,6]

Orthosiphon stamineus is an herbaceous plant and it has two types of flower, white and purple colour. This variety may cause the differences in chemical contents as well as biological activities.[7] The purple variety has more bioactive compounds than the white one. However, most scientific investigations have used the white variety.[8] Moreover, solvent, duration, temperature, and method of extraction as well as geographical location may also influence the chemical compounds of OS.[6, 9-13] Geographical origin of plants is important to be recognized since it affects the levels of bioactive compounds, a general criterion in the selection of raw materials. Therefore, a simple and accurate analytical method is needed to identify the geographical origin of OS.

Quality evaluation of OS crude drugs and its extract can be carried out using two approaches, the concentration of marker compound(s) and the profile of fingerprint. A groups of marker, rosmarinic acid (RA), 3′-hydroxy-5,6,7,4′-tetramethoxyflavone (TMF), sinensetin (SIN), and eupatorin (EUP) have been determined using HPLC[11] and HPTLC[13,14] methods to evaluate OS extract obtained from various origins, solvent, and method of extraction. Gas chromatography (GC) and FT-IR have been applied to develop the fingerprint of OS collected from various origins and prepared with different methods of extraction.[7,10-12]

Application of fingerprint reveals several superiorities compared to those of marker compounds. Fingerprint is a distinctive profile or pattern of sample which chemically reflects its composition in which as much information as possible is presented.[15,16] Therefore, almost all of compounds in a plant are considered in the analysis. This allows quality evaluation of a plant to be more objective than using only certain compound(s). TLC-fingerprint has been widely used in herbal medicines assessment. It is simpler, faster, and more cost effective compared to GC,

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HPLC, and FT-IR. TLC is able to analyze many samples in one running time. It is also possible to visually analyze TLC-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 on objective mathematical methods and treats the chromatogram as a unique signal, without a need to identify and interpret the peaks. Therefore, it provides a good possibility for mining more useful chemical information from original-rich data.[16-18] In this research we developed TLC-fingerprint combined with chemometrics to differentiate OS collected from various origins. Although several works on fingerprinting of OS have been reported before, none of them involved comparison between OS from different origins using TLC method.

MATERIALS AND METHODS

Chemicals

The following chemicals were procured from Merck (Darmstadt, Germany): TLC plate pre-coated with silica gel 60 F_{254}, n-hexane, diethyl ether, tetrahydrofuran, acetic acid, n-propanol, dichloromethane (DCM), ethyl acetate (EA), dioxane, toluene, chloroform ($CHCl_3$), methanol, ethanol, anisaldehyde, and sulfuric acid.

Plant materials

Orthosiphon stamineus were collected from eleven cultivation area in East and Central of Java, Indonesia (Table 1, Figure 1). Plant parts used were 5 leaves from the shoots that have not flowered yet. Leaves were harvested and cleaned with tap water. All samples were authenticated by Center for Traditional Medicine Information and Development, Faculty of Pharmacy, University of Surabaya, Indonesia (certificate number: 1400/D.T/V/2019). Leaves were then dried and ground into mesh 60.

Preparation of extracts

One gram of powdered *Orthosiphon stamineus* leaves was extracted with 10 ml of methanol, using Ultrasound-Assisted Extraction (UAE) method for 15 minutes. Extracts were subsequently filtered and kept in a tightly closed bottle.

TLC-fingerprint analysis

TLC condition

A Camag TLC system comprising of Linomat 5 sample applicator, twin-through chamber, and TLC-Visualizer with 12 bit CCD camera and Camag VideoScan 1.02 software serial number 2503D001 (Camag, Muttenz, Switzerland) were used. Chromatography was performed on Merck TLC plates (Art. No.: 1.05554.0001, silica gel 60 F_{254} pre-coated, 20 × 20 cm, 175-225 µm layer thickness, aluminium-backed, particle size distribution: 9.5-11.5 µm) with a 100-µl Camag syringe. Samples were spotted under a flow of nitrogen as 8 mm bands, 10 mm from the left edge, 15 mm from the bottom edge and 20 mm of track distance. Development was carried out in a chamber previously equilibrated (for 30 min at room temperature) with mobile phase (see: *selection of mobile phase*) and migration distance was 80 mm. The plates were dried under room temperature and then dipped in anisaldehyde-sulfuric acid reagent. TLC plates were then dried in fume hood and heated for 10 min at 100 °C. TLC plate were subsequently illuminated under short-wave UV (254 nm), long-wave UV (366 nm), and white light by using

Table 1: Geographical origins of *Orthosiphon stamineus* from Indonesia.

Code	District (City)	Height (mamsl)*	Latitude, Longitude	Time of collection
A	Lamongan	6	7°11' S; 112°42' E	May 2019
B	Pasuruan	800	7°41' S; 112°39' E	May 2019
C	Malang	445	7°43' S; 112°40' E	May 2019
D	Gresik	3	7°10' S; 112°40' E	May 2019
E	Surabaya	2	7°15' S; 112°42' E	May 2019
F	Sidoarjo	3	7°29' S; 112°43' E	May 2019
G	Jombang	44	7°20' S; 112°45' E	May 2019
H	Mojokerto 1	700	7°40' S; 112°36' E	May 2019
I	Karanganyar	1200	7°42' S; 111°80' E	February 2018
J	Batu	875	7°42' S; 112°42' E	February 2018
K	Mojokerto 2	700	7°40' S; 112°36' E	May 2019

*Metres above mean sea level

Figure 1: *Orthosiphon stamineus* from Lamongan (a), Pasuruan (b), Malang (c), Gresik (d), Surabaya (e), Sidoarjo (f), Jombang (g), Mojokerto 1 (h), Karanganyar (i), Batu (j), and Mojokerto 2 (k).

TLC-Visualizer. Quantitative evaluation of digitized images was carried out with Camag VideoScan 1.02 software.

Selection of the mobile phase

Ten ml of each solvent i.e. diethyl ether, 2-propanol, ethanol, tetrahydrofuran, acetic acid, dichloromethane, ethyl acetate, dioxane, toluene, and chloroform were put into a twin-through chamber and saturated for 30 minutes. Five µl of OS leaves extract was applied on TLC plate. The plate was then developed with the migration distance of 80 mm. After development, the plates were removed and dried at room temperature. In addition to the single mobile phase, the selection of mobile phase was also carried out using mixture of two or three solvents with various comparisons to obtain a chromatogram with the highest number and the best separations of zones.

Stability of the chromatograms

The stability of analytes in solution and on the plate was assessed by leaving the extract at room temperature and on the plate for 3 hours. As a standard, the new extract was prepared and applied just before the elution is started. The analytes were considered to be stable in the solution and on the plate before the chromatographic process if the difference of their Rf value is not more than 0.05. Stability of the analytes during chromatography was evaluated by two-dimensional (2D) elution. The analytes were concluded to be stable during the chromatographic process if all zones located on a diagonal connecting the initial position of the application with the intersection of the two mobile phase fronts. Moreover, to investigate the stability of the chromatographic result, the sample was chromatographed according to the method and derivatized with anisaldehyde-sulfuric acid reagent. The chromatogram was evaluated using TLC-visualizer repeatedly after 5, 10, 30, and 60 minutes. The chromatogram was concluded to be stable if there are no significant changes in Rf value.[19]

Precision of the chromatograms

Orthosiphon stamineus leaves were extracted in triplicate. Each extract was chromatographed three times on 3 different plates. Intraday precision was analyzed by calculating the Rf values of several prominent zones then calculating its mean and relative standard deviation. A reasonable acceptance criterion would be that Rf values of the same compounds do not vary more than 0.02 from plate to plate. To determine the interday precision, the extract was applied three times on 3 different plates and on 3 different days. Interday precision was acceptable if Rf values of the same substances vary not more than 0.05 between plates from different days.[19]

TLC of samples from different origins

OS leaves extracts from 11 different locations were chromatographed on a TLC plate. The TLC plate was then eluted as described before. TLC plate was subsequently illuminated under short-wave UV (254 nm), long-wave UV (366 nm), and white light by using TLC-Visualizer. Videodensitogram of samples were then analyzed using chemometric method.

Chemometric analysis

Rf value, height, and area of each peak (compound) obtained from videodensitogram was tabulated and then analyzed using Principal Component Analysis (PCA). PCA is an exploratory data 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[15]. PCA was carried out using Minitab v.16 (Minitab Inc., USA).

RESULTS AND DISCUSSION

Selection of the mobile phase

Methanol extracts of OS leaves collected from 11 origins are presented at Figure 2. All of extracts show dark green colour, except OS extract from Batu district (sample j). This is an initial indicator that sample from Batu may differ from the others. One of these extracts was then chosen for the mobile phase selection. At the first step, 10 single mobile phases were used and chloroform, dichloromethane, as well as ethyl acetate were the best mobile phase. These single mobile phases exhibited the most number and the best separation of zones on the TLC plate. These solvents were then mixed using simplex centroid with axial design.[20] Ten types of combination in different ratio were deduced and $CHCL_3$, DCM, EA (4:1:1) showed the optimum solvent system (Figure 3). However, the separation of zones with this solvent ratio was not so good, therefore modified ratios were applied i.e. 5:2:1 and 7:4:1. Finally, $CHCL_3$, DCM, EA in the ratio of 7:4:1 was then selected as a solvent system for the development of OS TLC fingerprinting. It exhibited 11 zones after derivatized with anisaldehyde-sulfuric acid reagent under white light.

Stability test

The stability of the analytes before and during the chromatography process is important to be established due to off-line nature of TLC system. The stability of the analyte before chromatography was determined by developing two extracts prepared at different times. The Rf values of the prominent zone or marker (*, yellowish-blue) at tracks I and II were compared to those at tracks III and IV to evaluate the stability of the analytes on the plate, while the zones at tracks V and VI were compared to those at tracks III and IV to assess the stability of the analytes in the solution.[19] The results showed that all tracks afforded the same pattern (Figure 4) and Rf value, with the $\Delta Rf < 0.05$ (Table 2). These indicated that the analytes remained stable for three hours before chromatography began, both on the plates and in the extract solution.

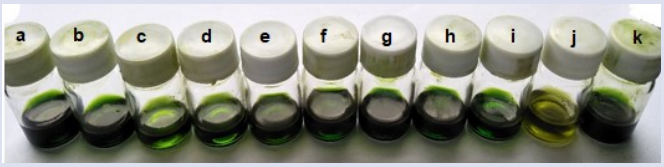

Figure 2: *Orthosiphon stamineus* leaves extracts from Lamongan (a), Pasuruan (b), Malang (c), Gresik (d), Surabaya (e), Sidoarjo (f), Jombang (g), Mojokerto 1 (h), Karanganyar (i), Batu (j), and Mojokerto 2 (k).

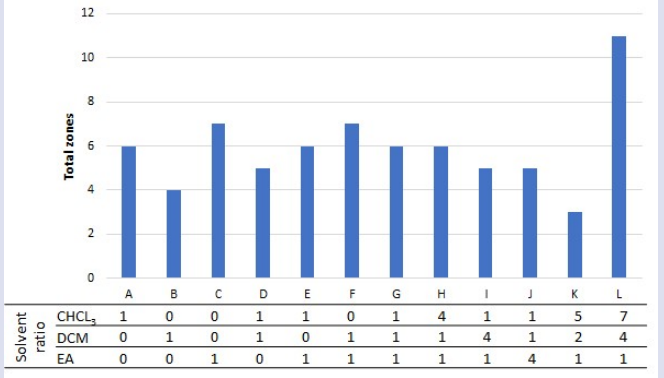

Figure 3: Total zones of OS leaves extract. Mobile phase (MP): $CHCL_3$, DCM, EA in different ratios. Detection (D): anisaldehyde-sulfuric acid reagent, white light.

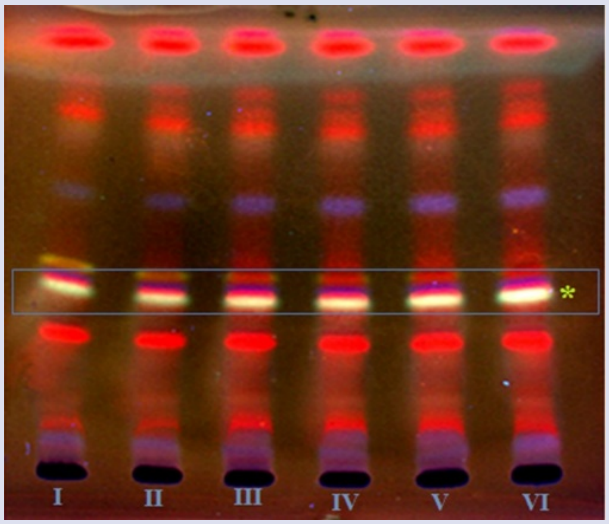

Figure 4: Stability of the analytes on plate and in solution. I, II: extracts on the plate for 3 hours; III, IV: extracts on the plate for less than 5 minutes; V, VI: extracts in solution for 3 hours. MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, UV 366 nm.

Table 2: Stability of analyte on the plate and in solution.

Marker (*)	Track I	Track II	Track III	Track IV	Track V	Track VI
Colour	Yellowish-blue	Yellowish-blue	Yellowish-blue	Yellowish-blue	Yellowish-blue	Yellowish-blue
Rf	0.418	0.394	0.388	0.384	0.391	0.403
Δ Rf[1]	0.032	0.008	0	0	0.005	0.017

[1]calculated to the marker compound at tracks III and IV

Two-dimensional (2D) development was carried out to determine the stability of the analytes during chromatography. A stable compounds will have the same Rf value in the first and second elution.[19] Figure 5 exhibited that the analytes were stable during the chromatographic process indicated by a diagonal line on 2D chromatogram, including marker compound (*).

Stability of the chromatographic results was determined by observing the color of the zones for 60 minutes. Figure 6 and Table 3 showed that the number and color of the zones were stable for 60 minutes. Differences of Rf value for the marker zone was not more than 0.05.

Precision test

Intraday precision test was conducted by chromatography of three OS leaves extracts prepared at the same time on one plate. Chromatography was replicated on 3 plates on the same day. Figure 7 and Table 4 show that the intraday precision of the method is acceptable since the ΔRf values of the marker zone (*) from plate to plate is 0.02.[19]

The interday precision test was carried out similar to those of intraday precision, however the development of plates I, II, and III was conducted on 3 different days. Figure 8 and Table 5 exhibited that the interday precision of the method is acceptable because the difference of the Rf value of marker (*) is 0.02.[19]

TLC-fingerprints of OS leaves from different origins

TLC profiles of OS leaves from 11 origins before and after derivatization are shown in Figure 9 and 10. Visually, sample 7 (Jombang) and 10 (Batu) showed significant differences compared to the others. Sample 7 has more zones and is more intensive compared to the others, while sample 10 is the opposite. This profile correlates with the colour of extract as presented at Figure 2.

TLC-fingerprint of samples after derivatization and visualized under UV 366 nm was then transferred into videodensitogram (Figure 11) to show their Rf value, height, and area. Total peak number of each sample varies between 27 and 34 peaks, all show major peak with Rf value 0.2-0.3. This peak can be further studied and can be considered as marker compound for OS. Moreover, height and peak area of sample 7 is more prominent compared to the others. This can be an initial indicator that the quality of sample 7 is better than the others. To compare the quality of samples more objectively, chemometric analysis was then performed on these TLC-fingerprints.

Principal component analysis (PCA)

Videodensitogram of OS leaves extracts from 11 different origins were analyzed with chemometric using PCA method. The height and area of each peak detected on the videodensitogram were then tabulated based on the origin of the sample (table is not shown). PCA with full cross validation was applied to the data set of the 11 fingerprints of OS from 11 origins, 3 replications respectively. Analysis was conducted

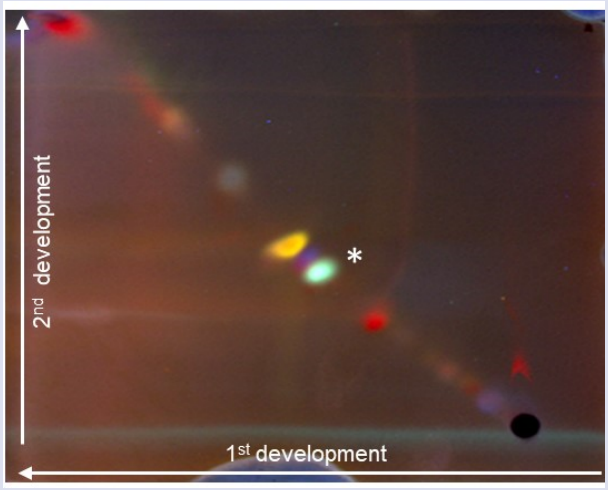

Figure 5: Stability of the analytes during chromatography. MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, UV 366 nm.

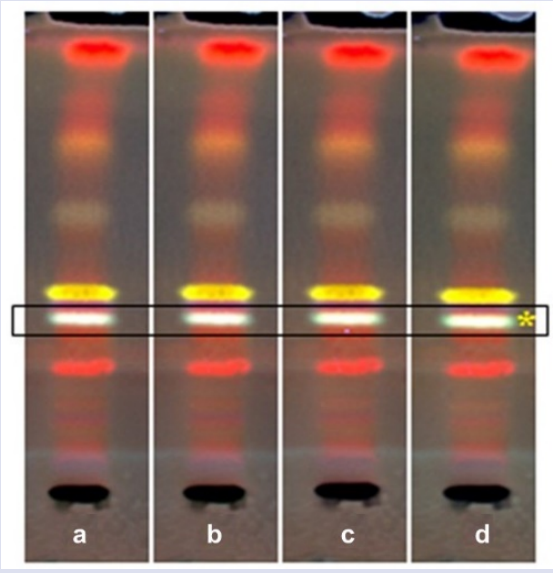

Figure 6: Stability of the chromatographic result after 5 (a), 10 (b), 30 (c), and 60 minutes (d). MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, UV 366 nm.

Table 3: Stability of the chromatographic result.

Marker (*)	Track a	Track b	Track c	Track d
Colour	Yellowish-blue	Yellowish-blue	Yellowish-blue	Yellowish-blue
Rf	0.367	0.366	0.376	0.378
Δ Rf[1]	0	0.001	0.009	0.011

[1]calculated to the marker compound at track a

Table 4: Intraday precision test.

Marker (*)	Track								
	I.1	I.2	I.3	II.1	II.2	II.3	III.1	III.2	III.3
Colour	Yellowish-blue			Yellowish-blue			Yellowish-blue		
Rf	0.404	0.388	0.388	0.431	0.409	0.406	0.415	0.402	0.393
Mean of Rf	0.39			0.41			0.40		
Δ Rf				0.02					

Table 5: Interday precision test.

Marker (*)	Track								
	I.1	I.2	I.3	II.1	II.2	II.3	III.1	III.2	III.3
Colour	Yellowish-blue			Yellowish-blue			Yellowish-blue		
Rf	0.404	0.388	0.388	0.431	0.409	0.406	0.420	0.377	0.367
Mean Rf	0.39			0.41			0.38		
Δ Rf				0.02					

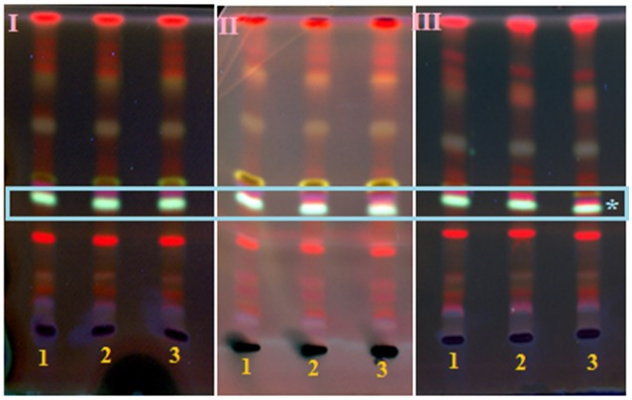

Figure 7: Intraday precision test. 1, 2, 3 are OS leaves extracts prepared separately, while I, II, III show the different plates. MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, UV 366 nm.

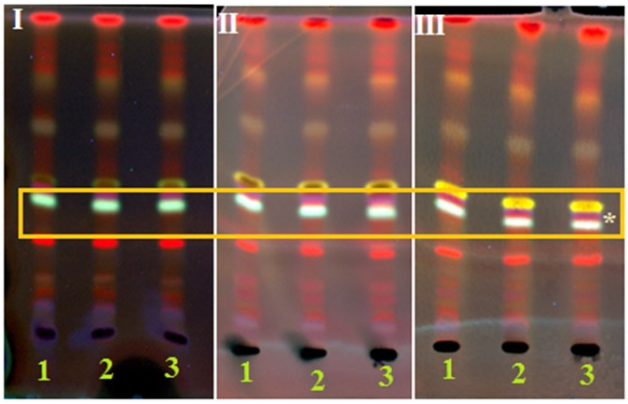

Figure 8: Interday precision test. 1, 2, 3 are OS leaves extracts prepared separately, while I, II, III show the plates developed in 3 different days. MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, UV 366 nm.

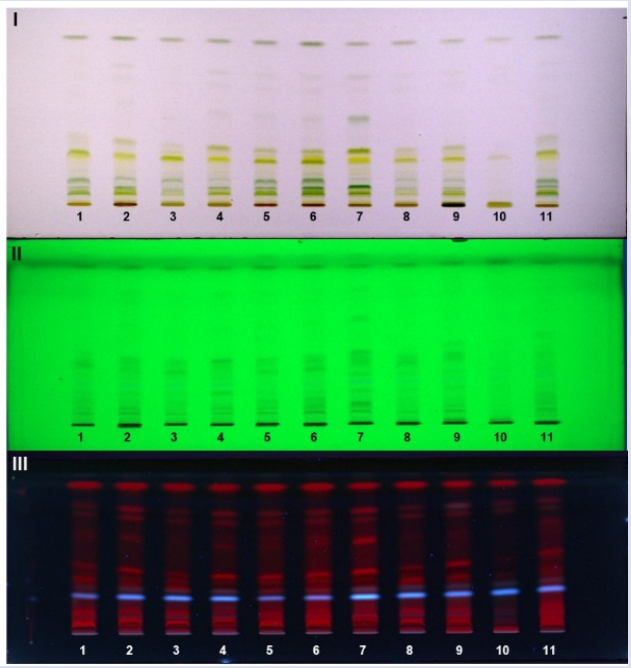

Figure 9: TLC-fingerprint OS leaves from Lamongan (1), Pasuruan (2), Malang (3), Gresik (4), Surabaya (5), Sidoarjo (6), Jombang (7), Mojokerto (8), Karanganyar (9), Batu (10), and Mojokerto (11). MP: $CHCL_3$, DCM, EA (7:4:1). D: none, white light (I), UV 254 nm (II), UV 366 nm (III).

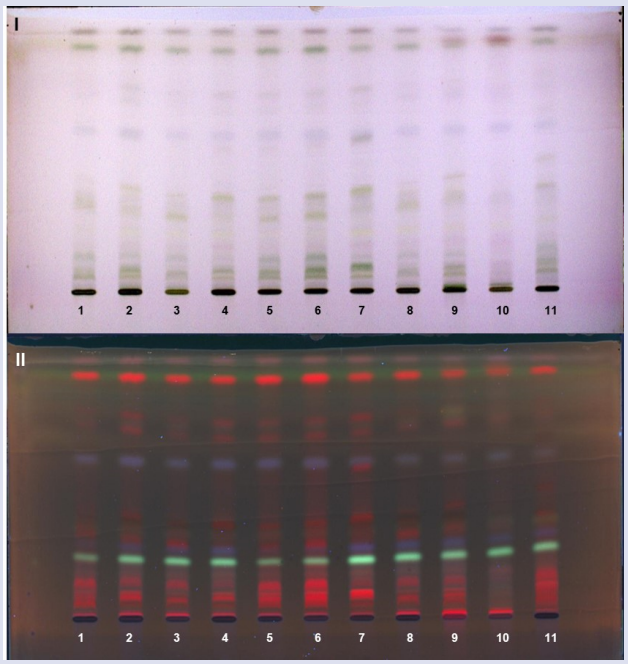

Figure 10: TLC-fingerprint of OS leaves from Lamongan (1), Pasuruan (2), Malang (3), Gresik (4), Surabaya (5), Sidoarjo (6), Jombang (7), Mojokerto (8), Karanganyar (9), Batu (10), and Mojokerto (11). MP: $CHCL_3$, DCM, EA (7:4:1). D: anisaldehyde-sulfuric acid reagent, white light (I), UV 366 nm (II).

on the peak height and area of the full fingerprints without any preprocessing. The score plot of the first two PC (Figure 12) clearly distinguished 3 clusters of samples. The first cluster consists of OS from Mojokerto 1, Mojokerto 2, Malang, Jombang, and Sidoarjo. OS from Gresik, Pasuruan, Lamongan, Surabaya, and Karang Anyar gathered as second cluster, whereas third cluster consists of a sample from Batu. Samples in the cluster 1 show that the compounds with Rf value 0.2-0.3 are higher than those in clusters 2 and 3. However, these results indicate that the clustering of OS samples is not directly related to the height of the original location. Other factors such as soil type, rainfall, lighting, fertilizing, etc. are predicted to be responsible for the samples grouping.[21]

To estimate which compounds are responsible for the grouping, PCA was conducted by projecting each origin variable called as loading plot. The loading plot of the first PC (Figure 13) showed that compounds with the Rf values of 0.0-0.1, 0.1-0.2, 0.2-0.3, and 0.9-1.0 are the most important compounds for clustering of samples.

Previous studies have been carried out to classify OS from different geographical origin. They involved both chromatography (HPLC, GC)

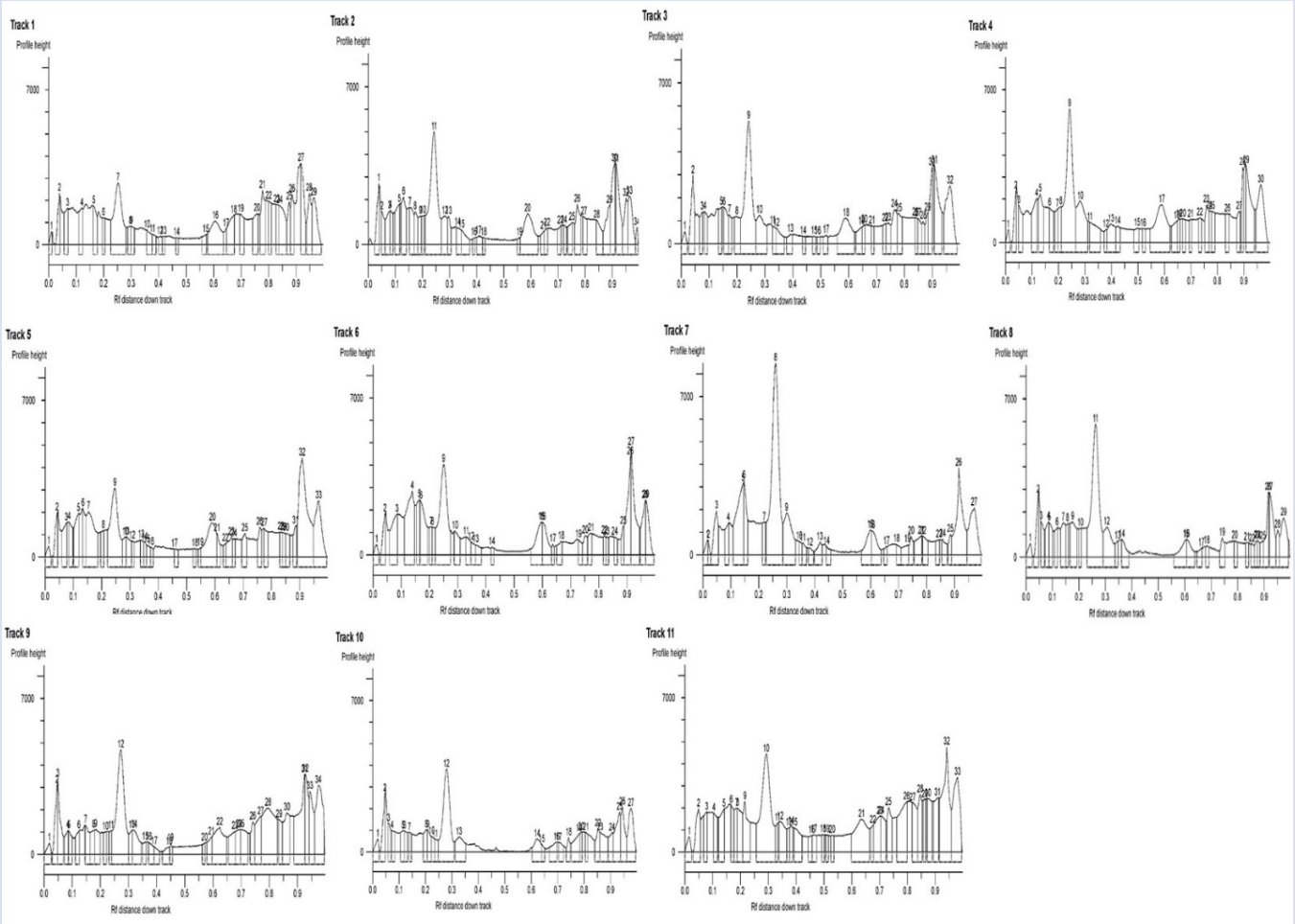

Figure 11: Videodensitogram of OS leaves, track 1-11 represent samples from Lamongan, Pasuruan, Malang, Gresik, Surabaya, Sidoarjo, Jombang, Mojokerto, Karanganyar, Batu, and Mojokerto, respectively.

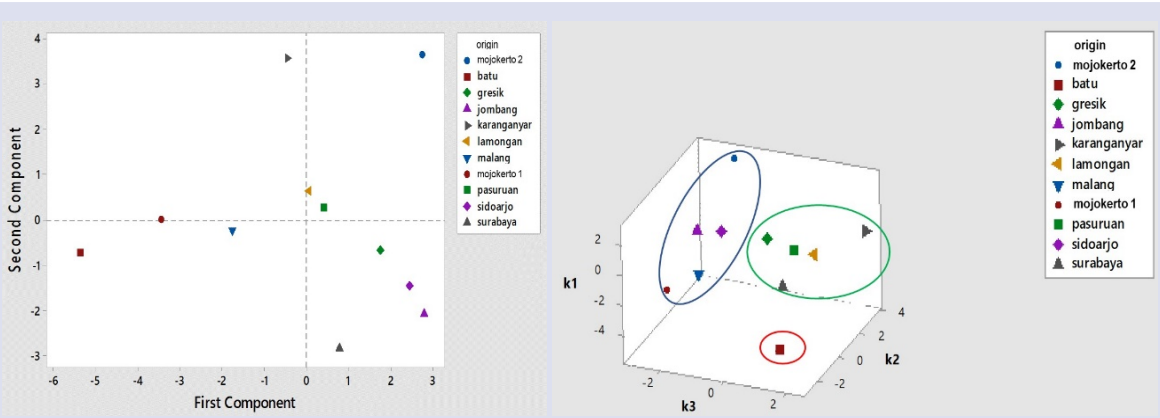

Figure 12: PCA score plot of OS from different origins on the first two principal components.

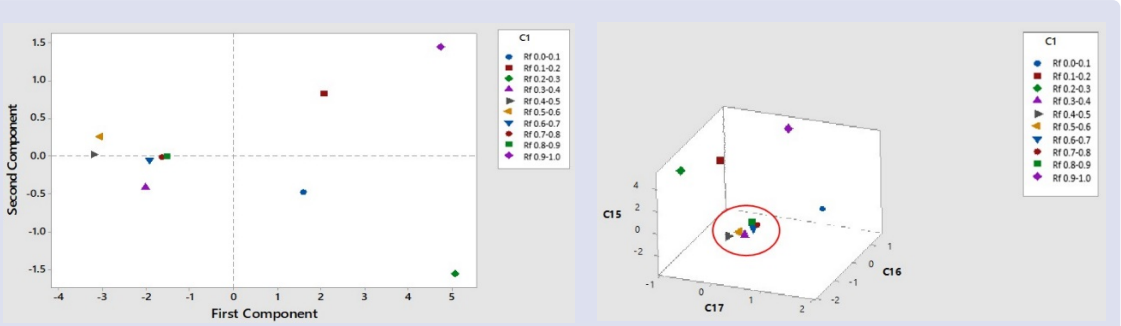

Figure 13: PCA loading plot of OS from different origins on the first two principal components.

as well as spectroscopy (FTIR) method coupled with chemometrics analysis.[10-12] Our finding suggested that TLC fingerprint coupled with chemometrics analysis is a good alternative method to HPLC, GC, and FTIR for quality control of OS as well as the other crude drugs. In addition to its simplicity and low cost, by using TLC we can simultaneously analysis up to 20 samples under identical conditions.

CONCLUSION

TLC-fingerprint combined with the PCA was able to discriminate among the leaves of *Orthosiphon stamineus* originated from various locations. Compounds with the Rf values 0.0-0.1, 0.1-0.2, 0.2-0.3, and 0.9-1.0 are the most important compounds for the clustering of samples. TLC-fingerprints analyzed with chemometrics is powerful method to evaluate the quality of *Orthosiphon stamineus*.

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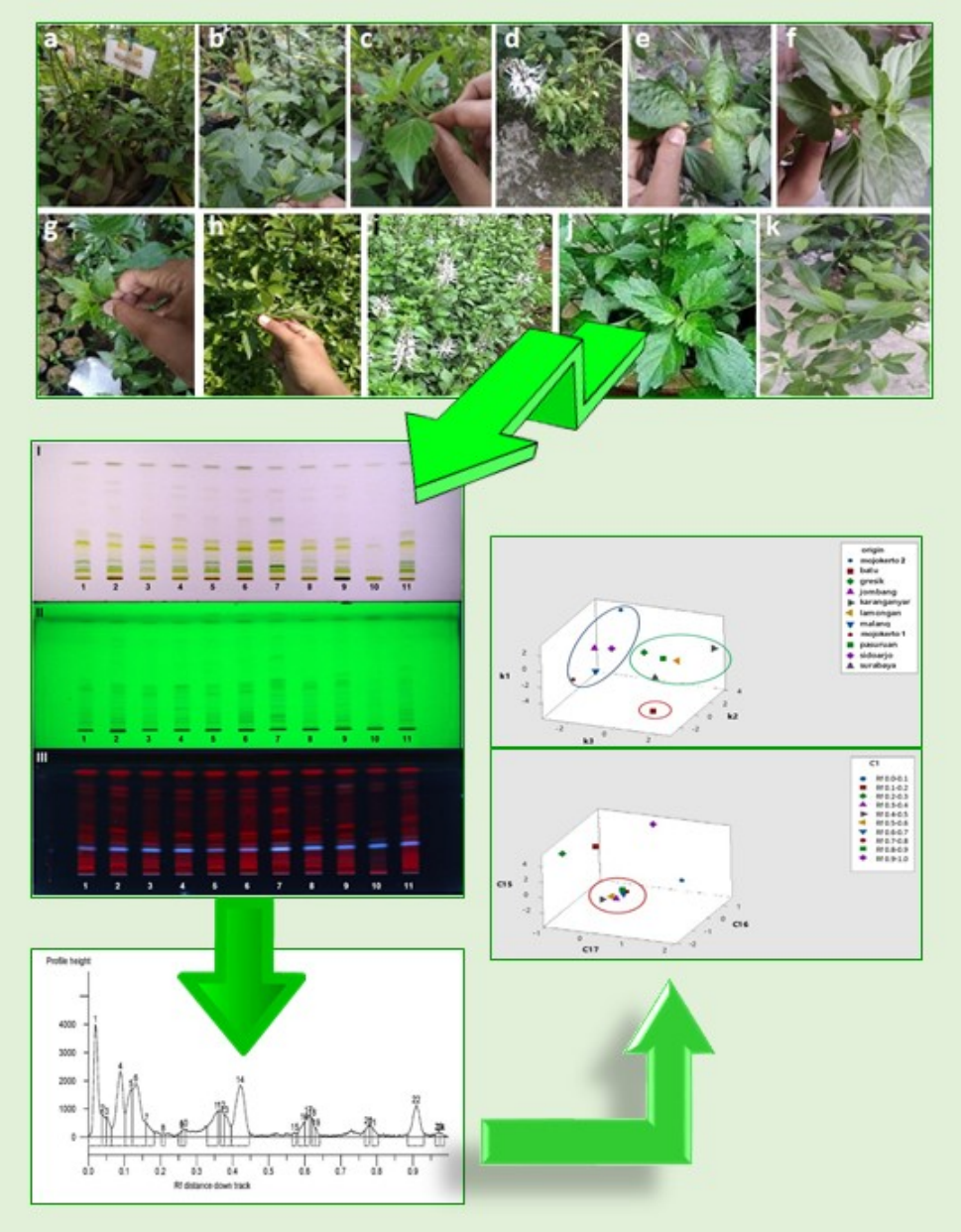
GRAPHICAL ABSTRACT



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