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TLC-fingerprinting and chemometrics for identification of *Curcuma xanthorrhiza* from different geographical origins in Indonesia

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Abstract. *Kartini K, Sabatini SS, Haridsa NM, Jayani NIE, Setiawan F, Hadiyat MA. 2023. TLC-fingerprinting and chemometrics for identification of* Curcuma xanthorrhiza *from different geographical origins in Indonesia. Biodiversitas 24: 6557-6566.* Geographical origin is an important parameter that influences the quality of herbal medicine. The fingerprint of herbal plants is expressed as chemical compounds contributing to said quality. *Curcuma xanthorrhiza*, popular as java turmeric, is extensively used in Indonesian traditional medicine. This study aimed to validate and develop a Thin Layer Chromatography (TLC) fingerprint of *C. xanthorrhiza*, followed by chemometric techniques to classify *C. xanthorrhiza* rhizomes from 15 regions in Indonesia. Under selected TLC conditions (i.e., stationary phase: TLC plate Silica gel 60 GF_{254}, mobile phase: dichloromethane, chloroform, and ethanol (10: 10: 1), detection: vanillin-sulfuric acid reagent), *C. xanthorrhiza* produced five zones of the compound. Extracts were stable both on the TLC plate and in the extract solution during chromatography and within 60 minutes after derivatization. In terms of precision, the chromatogram meets the requirement of intraday precision. Chemometric analysis with Principal Component Analysis (PCA) and Cluster Analysis (CA) showed that the samples of *C. xanthorrhiza* from 15 regions in Indonesia were grouped into five clusters based on the similarity of the chemical compounds, namely cluster 1 (Tawangmangu, Bangkalan, Kediri, and Surabaya); cluster 2 (Batu, Sragen, Tulungagung, Pasuruan, Blitar, and Lombok Tengah); cluster 3 (Ngawi); cluster 4 (Gresik); and cluster 5 (Bojonegoro, Banyuwangi, and Palangkaraya). The TLC fingerprinting with chemometrics on *C. xanthorrhiza* rhizome is useful for quality control based on geographic origin and authenticity identification.

Keywords: Chemometrics, *Curcuma xanthorrhiza*, discriminant analysis, geographic origin, java turmeric, TLC-fingerprinting

INTRODUCTION

Curcuma xanthorrhiza belongs to the Zingiberaceae family and is better known as java turmeric, a native Indonesian herb originating in Java, Bali, and Maluku islands. The rhizome is the widely used part of the plant with yellowish to reddish peel and orange to reddish-orange inside. It has two main active compound groups: curcuminoids and bisabolene sesquiterpenes (Rahmat et al. 2021). Curcuminoids in java turmeric consist of curcumin, demethoxycurcumin, and bisdemethoxycurcumin; curcumin is the most abundant (Canistro et al. 2021). Meanwhile, xanthorrhizol is a bisabolene sesquiterpene that acts as the primary bioactive constituent of *C. xanthorrhiza* (Nurcholis et al. 2018).

Curcuminoids are a class of phenolic compounds exhibiting high antioxidant activity in various in vitro and in vivo tests (Nurrulhidayah et al. 2020; Lukitaningsih et al. 2020; Rosidi 2020). Meanwhile, the activity of xanthorrhizol is thought to be related to its anticancer and antihyperglycemic activity (Kim et al. 2013; Kim et al. 2014). Ethnomedicinally, in Indonesia, *C. xanthorrhiza* is used for stomach aches, epilepsy, tonsillitis, cough, dysentery, bone pain, fever, etc. (Subositi and Wahyono 2019; Pramono et al. 2018).

Curcuma xanthorrhiza is one of the country's most extensively used medicinal plants; therefore, its cultivation is in great demand. Data from 2019 shows that *C. xanthorrhiza* is massively cultivated in Indonesia, with a planting area of 1,300 hectares, producing nearly 30,000 tons of rhizomes (Rahmat et al. 2021). With such a high consumption rate, adulteration with several other similar rhizomes is highly likely, e.g., *Curcuma longa*, *Zingiber montanum*, and *Curcuma zedoaria* (Rafi et al. 2011; Angeline et al. 2019; Windarsih et al. 2021). Generally, herbal ingredients often face another problem: varying qualities caused by differences in variety, geographical origin, climate, harvest season, and storage conditions (Yudthavorasit et al. 2014). Previous research reported that *C. xanthorrhiza* collected from two different locations in Indonesia had different levels of curcumin and antioxidant activity (Rosidi 2020). This proves that the quality of this rhizome is influenced by its geographical origin.

There are some methods for material analysis of herbal medicine products, including UV spectroscopy and IR spectroscopy using chemical fingerprinting and chemical markers approaches (Liu et al. 2018). A method that can be used to authenticate and identify the quality of *C. xanthorrhiza* is by creating a fingerprint profile of the plant using chromatography and spectroscopy techniques. Some scholars have created fingerprint profiles of *C. xanthorrhiza* using thin-layer chromatography (TLC) (Rafi

et al. 2011; Rohman et al. 2020), proton nuclear magnetic resonance (^{1}H-NMR) (Windarsih et al. 2021; Rohman et al. 2020), Fourier transform infrared (FTIR) spectroscopy (Angeline et al. 2019), and high-performance liquid chromatography (HPLC) (Rafi et al. 2015).

Chromatographic fingerprint analysis has been accepted by the World Health Organization (WHO), Food Drug Analysis (FDA), and European Medicines Agency (EMEA) for herbal sample identification and quality control (Rafi et al. 2011; Hawrył et al. 2016; Hawrył et al. 2020; Liu et al. 2016). TLC fingerprinting is particularly selected because it is effective, simple, easy to apply, time-efficient, and has a low cost of analysis (Hawrył et al. 2016; Hawrył et al. 2019). TLC-based fingerprint profiles are multivariate and, thus, require a special technique for their analysis, i.e., chemometrics. Chemometric methods, especially Principal Component Analysis (PCA) and Cluster Analysis (CA) have been used extensively for various purposes (e.g., distinguishing species and plant origin) owing to their ability to analyze the chemical profile of herbs objectively (Ma et al. 2018; Sun et al. 2018; Tan et al. 2018; Huang et al. 2018; Shen et al. 2019). This study was designed to develop TLC-based fingerprint profiles of *C. xanthorrhiza*, followed by applying chemometrics to distinguish *C. xanthorrhiza* rhizomes collected from several regions and classify them according to shared chemical profiles.

MATERIALS AND METHODS

Chemicals and instrumentation

Curcuma xanthorrhiza rhizome samples were collected from 15 cultivation sites in Indonesia, namely from the provinces of Jawa Tengah, Jawa Timur, Kalimantan Tengah, and Nusa Tenggara Barat (Table 1) and identified by the Center for Information and Development of Traditional Medicine, Universitas Surabaya, Indonesia, with a certificate of plant identification No. 1435/D.T/I/2021. Sample preparation for further analysis started with wet sorting, where the *C. xanthorrhiza* rhizomes were removed and then separated from impurities, such as soil. The following stages were washing, draining, chopping, drying, and dry sorting. Afterward, the dry samples were ground and passed through a 60-mesh sieve (Figure 1).

Table 1. Geographical origin of *C. xanthorrhiza* rhizomes from Indonesia

District	Height (masl)	Latitude, Longitude
Tawangmangu	966	7°39'48.7"S, 111°08'08.3"E
Batu	897	7°52'01.0"S, 112°31'11.6"E
Sragen	128	7°24'20.1"S, 110°51'11.3"E
Tulungagung	85	8°13'26.8"S, 111°51'57.5"E
Bangkalan	5	7°02'27.1"S, 112°43'51.0"E
Ngawi	84	7°29'25.6"S, 111°26'36.9"E
Bojonegoro	16	7°09'00.2"S 111°52'32.6"E
Gresik	11	7°20'01.1"S, 112°38'03.6"E
Pasuruan	25	7°38'12.7"S, 112°47'37.1"E
Banyuwangi	110	8°09'03.7"S, 114°20'43.5"E
Surabaya	7	7°17'52.2"S, 112°44'12.3"E
Kediri	84	7°41'15.2"S, 112°11'43.2"E
Blitar	105	8°03'34.3"S, 112°00'37.6"E
Palangkaraya	15	2°15'48.1"S, 113°55'13.8"E
Lombok Tengah	166	8°48'15.8"S, 116°22'54.0"E

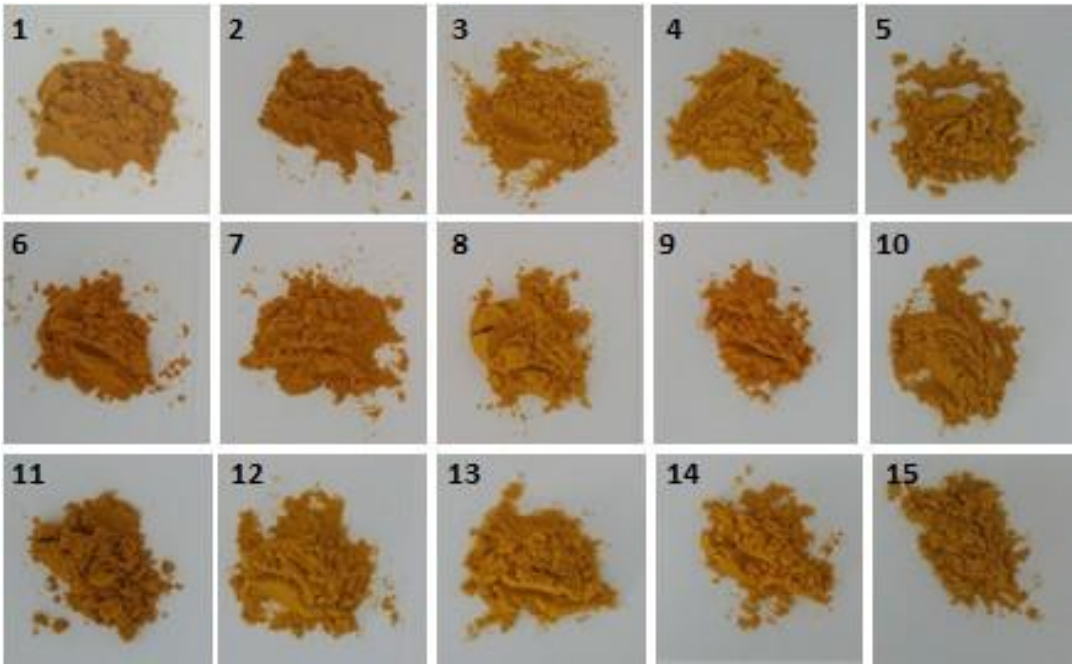

Figure 1. *Curcuma xanthorrhiza* crude drug powders collected from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15)

Chemicals used in this study are produced by Merck (Darmstadt, Germany) and consist of TLC silica gel 60 GF_{254} plates, anisaldehyde, vanillin, glacial acetic acid, sulfuric acid, chloroform ($CHCl_3$), tetrahydrofuran, ethanol (C_2H_5OH), methanol, dioxane, n-hexane, toluene, ethyl acetate, acetic acid, 2-propanol, diethyl ether, and dichloromethane (CH_2Cl_2). All solvents are analytical reagent grade. Curcumin (≥65%, HPLC) is from Sigma-Aldrich (Missouri, US). The instruments used in this study were a twin-trough-chamber chromatography chamber (Camag, Muttenz, Switzerland), oven (Memmert, Schwabach, Germany), analytical balance (Ohaus, Schwabach, Germany), semiautomatic Linomat 5 TLC sampler (Camag), Mettler Toledo moisture analyzer, 254 nm and 366 nm UV light detector (Camag), TLC Visualizer with 12 bit CCD camera and Camag VideoScan 1.02 software with the serial number 2503D001, and ultrasonic bath (US ONE, Osaka, Japan).

Procedures

Extract preparation

The preparation of *C. xanthorrhiza* extract referred to the previous research with slight modification (Kartini et al. 2021). A dry sample of *C. xanthorrhiza* powder weighing 0.5 g was extracted using 10 ml of ethanol in an ultrasonic bath (42 kHz) for 15 minutes at room temperature. The resulting extract was filtered and put in a tightly closed glass bottle. This procedure was done for all 15 samples of *C. xanthorrhiza*.

Method of validations

TLC conditions

The chromatographic conditions used in this study were adopted from the previous research, which was conducted on turmeric with some modifications (Kartini et al. 2021). A sample of the *C. xanthorrhiza* extract (6 µL) and a curcumin analytical standard (0.1%, 4 µL) were sprayed using a semiautomatic TLC sampler onto the silica gel 60 GF_{254} plate in the form of 7 mm-wide bands placed with a distance of 4 mm, 15 mm from the bottom side, and 14 mm from the left and right sides of the plate. The plate was then eluted in a chamber saturated with mobile phase (as stated in the section of *Mobile phase selection*) for ± 30 minutes. After the eluent traveled 80 mm upward, the plate was pulled out of the chamber and left to dry at room temperature. Then, it was dipped in the vanillin-sulfuric acid reagent to stain the spots and dried in an oven (100° C) for ten minutes. The plate was observed using a TLC Visualizer under visible light, UV 254 nm, and UV 366 nm, and the complete chromatogram was evaluated quantitatively in the Camag VideoScan 1.02 program.

Mobile phase selection

An optimal mobile phase selected for the TLC fingerprint is the one that can produce the largest number of bands (compound stains) with the best separation. The selection process was conducted in two stages: selecting a single mobile phase (a solvent) and creating a mixture of solvents (Borges et al. 2007). In the first stage (a single solvent), a sample of the *C. xanthorrhiza* extract was eluted with different solvents individually, namely $CHCl_3$, tetrahydrofuran, C_2H_5OH, dioxane, n-hexane, toluene, ethyl acetate, acetic acid, 2-propanol, diethyl ether, and CH_2Cl_2. Then, in the second stage (a mixture of solvents), two to three optimal mobile phases determined in the first stage were mixed to obtain the suitable mobile phase composition for TLC fingerprinting.

Stability test

The chromatogram indicates three components of analyte stability: in the extract solution and on the TLC plate, during chromatography, and after derivatization. The stability test method referred to Reich and Schibli (Reich and Schibli 2007) with modifications. The stability of the analyte in the extract solution and on the TLC plate was tested in parallel using a 10 x 10 cm TLC plate. Several analyte samples, i.e., *C. xanthorrhiza* extracts, were prepared (see the *Extract preparation* section). The TLC plate was first spotted with the fresh extract in columns 1 and 2 and stored at room temperature for three hours. Then, it was spotted with the same extract in columns 5 and 6. Afterward, another freshly made extract was immediately applied to the same plate in columns 3 and 4 right before the elution. A chemical compound is stable if its Rf value in the extract solution and plate does not differ by more than 0.05 from the reference Rf.

The stability of the analyte during chromatography can be observed by developing a 2D chromatogram. The sample solution was spotted on the 10 x 10 cm TLC plate at the lower right corner (at least 10 mm from the edge). The plate was then eluted with the appropriate mobile phase. After the first elution, it was dried, rotated 90° counterclockwise, and re-eluted using a new mobile phase. A sample is stable during chromatography if the zones form a diagonal line connecting the initial point (lower right corner) and the intersection of the two elution results (Reich and Schibli 2007).

After derivatization, the analyte's stability was assessed by spotting the sample on a 10 cm x 10 cm plate and then eluting it with the selected mobile phase. The vanillin-sulfuric acid reagent was sprayed onto the plate, then the results were observed after 5, 10, 30, and 60 minutes. An analyte is considered stable after derivatization if the Rf value does not vary throughout the 60-minute observation (Reich and Schibli 2007).

Precision of chromatogram

A sample of *C. xanthorrhiza* rhizome was extracted in triplicates, and each was applied twice on three different TLC plates. Precision was analyzed by calculating the Rf values of each zone and the mean Rf and standard deviation. A chromatogram is classified as precise if the fingerprint patterns resulting from the precision test (comprising number, location, color, the color intensity of the zone, and Rf difference of not more than 0.02) are identical (Reich and Schibli 2007).

TLC-fingerprint and chemometric analysis

The extracts of the *C. xanthorrhiza* rhizome samples collected from 15 areas in Indonesia were applied to TLC

plates according to the previously optimized conditions. Each sample's TLC-derived fingerprint profile/TLC profile was converted into a video densitogram containing each compound peak's Rf value, height, and area. Afterward, these data were tabulated, with sample origin presented in rows and peak height at certain Rf in columns, and analyzed using a chemometric method, i.e., Principal Component Analysis (PCA) and Cluster Analysis (CA). PCA reduces the complexity of multivariate data sets by explaining the correlations among a certain number of variables with smaller underlying factors termed Principal Components (PCs) without losing information. Projections of the original data on PCs are called score plots, where the contribution of each original variable to the score is shown by its weight (loadings) to detect which variable is responsible for or can explain the clustering (Tistaert et al. 2011). In CA, samples were grouped into clusters based on the distance between the cluster members; then, the results were presented in a dendrogram (Ma et al. 2018) and analyzed with a chemometric method in the Minitab v.16 program (Minitab Inc., USA).

RESULTS AND DISCUSSION

Physical characteristics of extracts

The ethanol extracts of *C. xanthorrhiza* rhizomes from 15 regions in Indonesia are generally yellow (Figure 2). The rhizome extracts from Batu, Ngawi, and Lombok Tengah appeared darker (brownish-yellow) than the others, which seems to correlate with the more brownish color of their rhizome powder (Figure 1). Different colors of powders and extracts can be used as an early indicator that the chemical content of the rhizomes from these three locations is different from other samples.

Selected mobile phase

The Tawangmangu rhizome extract was used for the optimization of the mobile phase. In the first stage, it was found that CH_2Cl_2, $CHCl_3$, and C_2H_5OH were single mobile phases producing the highest number of bands (compound stains) with the best separation, i.e., 2 bands. The second stage combined these mobile phases using a mixture design, i.e., a simple centroid with an axial design. A mixture of two or three solvents with different polarities is required since the sample also contains various compounds with different polarities. However, because this method did not show which mixture of mobile phases produced an optimal chromatogram profile, the composition ratios of the mixture were slightly modified. Based on the chromatography tests of the 13 mixtures, the combination of CH_2Cl_2, $CHCl_3$, and C_2H_5OH with a ratio of 10: 10: 1 produced 5 zones after derivatization by vanillin-sulfuric acid reagent and visualization under UV 366 nm, meaning that it is the optimal mixture of mobile phase for the TLC fingerprinting of *C. xanthorrhiza* rhizomes.

Chromatogram stability

Although TLC proves effective for compound fingerprinting, it is an open system and cannot be entirely conducted online. Compared to other chromatography techniques, this disadvantage can change air, light, smoke, dust, temperature, and other factors and potentially degrade the sample on the plate surface during the process. For these reasons, it is necessary to ensure the stability of the analyte throughout the analysis stages: on the plate and in the extract solution, during chromatography, and after derivatization.

The stability of the analyte during chromatography was determined by developing a 2D chromatogram from two-time elution. Figure 3.A shows that the zones produced in the first and second elutions formed an imaginary diagonal line, indicating that the Rf values of these zones are identical. An analytical sample is stable during chromatography if all the zones lie on a diagonal line connecting the intersection of the solvent fronts. Meanwhile, a zone away from the diagonal line indicates that the compound is unstable during chromatography (Reich and Schibli 2007).

The stability of the analyte on the plate and in the extract solution was determined by comparing the Rf values and the colors of the analyte zones in columns 1 and 2 (samples were spotted on the plate three hours before elution) with those of the analyte in columns 3 and 4 (freshly made samples were spotted immediately, just before the elution). In addition, the Rf values and the colors of the analyte zones in columns 5 and 6 (freshly made samples from the previous spotting were left three hours and spotted just before the elution) were compared with those of the analyte in columns 3 and 4. The analyte's compound is stable if its Rf values in the extract solution and on the plate do not differ by more than 0.05 from the reference Rf (Reich and Schibli 2007). As shown in Figure 3.B, the analyte sample was stable both in the extract solution and on the plate for three hours. Further, Table 2 shows the colors and Rf values of the *C. xanthorrhiza* rhizome extracts in this analyte stability testing. The ΔRf values in columns 1-6 were less than 0.05, thus indicating that the samples have good stability on the TLC plate and in the extract solution.

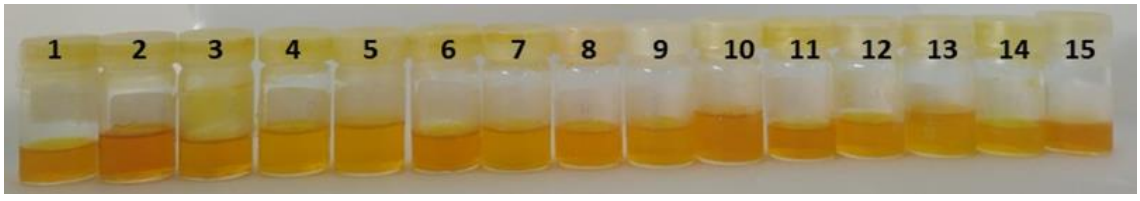

Figure 2. Ethanol extract of *C. xanthorrhiza* from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15)

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

Marker (*)	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Color	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence
Rf values	0.297	0.297	0.297	0.297	0.317	0.339
ΔRf[a]	0	0	0	0	0.02	0.042

Note: [a]Compared to markers in columns 3 and 4

Table 3. Stability of chromatogram after derivatization

Marker (*)	5 min (a)	10 min (b)	30 min (c)	60 min (d)
Color	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence
Rf values	0.347	0.349	0.345	0.340
ΔRf[a]	0	0.002	0.002	0.007

Note: [a]Compared to the marker at 5 min

Table 4. Intraday precision

Marker (*)	a	b	c
Color	Yellow fluorescence	Yellow fluorescence	Yellow fluorescence
Rf Values	0.35	0.34	0.37
ΔRf		0.02	

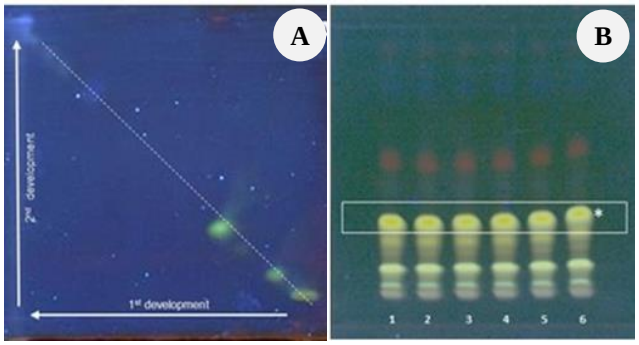

Figure 3. A. The stability of the analyte during chromatography, **B.** The stability of the analyte on the plate and in the extract solution. **1, 2:** extract was spotted on the plate and stored at room temperature for three hours, **3, 4:** freshly made extract was immediately applied to the plate right before the elution, **5, 6:** extract was stored at room temperature for three hours and the spotted on the plate. MP (mobile phase): CH_2Cl_2, $CHCl_3$, and C_2H_5OH (10: 10: 1). D (detection): vanillin-sulfuric acid reagent, UV 366 nm. (*) marker compound

After derivatization, a sample is considered stable in the stability test if its chromatogram profile does not change over a predefined period after spraying and reacting with the staining reagent. As seen in Figure 4 and Table 3, there was no significant temporal change in the chromatogram profile. Besides, no zones appeared or disappeared within 60 minutes, and the ΔRf values did not exceed 0.05. All of these indicate that the *C. xanthorrhiza* rhizome extracts are stable during derivatization (Reich and Schibli 2007).

Precision of the chromatogram

Moreover, apart from having an open and partially online-operated system, TLC is semiautomatic and can lead to a less precise chromatogram profile than other chromatography techniques, such as high-performance liquid chromatography (HPLC). Therefore, precision is an important validation parameter to ensure before TLC fingerprinting. A TLC system is precise if the chromatogram profiles it produces have an identical number of zones, location, and color intensity and if the ΔRf values from three different plates where the samples were eluted under the same conditions on the same day do not exceed 0.02 (Reich and Schibli 2007). Figure 5 and Table 4 show that the TLC system used for fingerprinting the *C. xanthorrhiza* rhizome extracts meets the precision aspect. This is evidenced by the Rf value of less than 0.02 on the three plates.

TLC fingerprints of *C. xanthorrhiza* collected from different origins

The ethanolic extracts of *Curcuma xanthorrhiza* rhizome collected from 15 different origins in Indonesia were applied to TLC plates according to the previously validated conditions. The chromatograms are presented in Figure 6. It can be seen that each sample shows a different pattern (number of bands and color intensity).

Figure 7 shows a video densitogram of the 15 *C. xanthorrhiza* rhizome samples containing information on each compound peak's Rf value, height, and area, allowing objective analysis of the TLC results. The number of peaks detected in the video densitogram varied, averaging 21 and 3 major peaks in the Rf ranges of 0.6-0.7, 0.8-0.9, and 0.9-1.0. Furthermore, the Rf values and peak heights from each sample were used for further chemometric analysis using Principal Component Analysis or PCA (data table not shown); these data were prepared in triplicates and spotted on three different TLC plates.

Principal component analysis (PCA) and cluster analysis (CA) results

The results of PCA and CA on 15 *C. xanthorrhiza* rhizomes can be seen in Figures 8 and 9, respectively.

Discussion

Curcumin is often used as a marker compound by several pharmacopeias and pharmaceutical industries (Health IMO 2017; Gupta et al. 2013). More than 65 clinical trials on curcumin have been conducted, and not less than 35 other clinical trials are ongoing to evaluate further its activities (Gupta et al. 2013). The curcuminoids, belonging to the diarylheptanoids group, comprise curcumin, desmethoxycurcumin, and bisdemethoxy-curcumin and are responsible for the yellow-to-orange color of the rhizomes. In addition to curcuminoids, xanthorrhizol is another component of *C. xanthorrhiza* rhizomes. Previous scholars have proved various biological activities of xanthorrhizol (Kim et al. 2013; Kim et al. 2014). Therefore, there is no doubt that the evaluation of C. *xanthorrhiza* will be more objective using chemical fingerprints rather than marker (s). Video densitogram (Figure 7) shows that *C. xanthorrhiza* rhizome samples had an average of 21 peaks, indicating that at least 21 chemical components were detected using the TLC method in each sample.

Among 21 compounds, it can be seen that curcuminoids were visible under white light before derivatization (Figure 6, IA). The marker compounds of curcuminoids (column 1) had different Rf values for curcumin (0.838), desmethoxycurcumin (0.631), and bisdemethoxycurcumin (0.400). Bisdemethoxycurcumin appeared most distinctly as a yellow zone in the Gresik rhizome sample (column 8, Figure 6, IA and IB). However, this marker compound was not detected in the samples collected from Tawangmangu, Bangkalan, Blitar, and Lombok Tengah (columns 1, 5, 13, and 15). This corresponds to Cahyono et al. 2019, which detected bisdemethoxycurcumin in minute or even undetectable amounts in *Curcuma xanthorrhiza* and identified this compound as the main differentiator between curcuminoids in *C. xanthorrhiza* and *C. longa*.

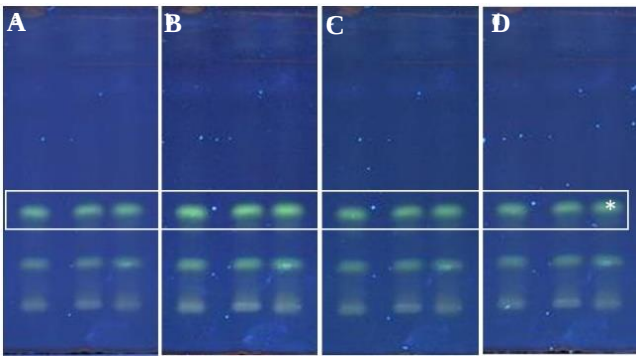

Figure 4. The stability of the analyte was 5 min (a), 10 min (b), 30 min (c), and 60 min (d) after derivatization. MP: CH_2Cl_2, $CHCl_3$, and C_2H_5OH (10: 10: 1). D: vanillin-sulfuric acid reagent, UV 366 nm. (*) marker compound

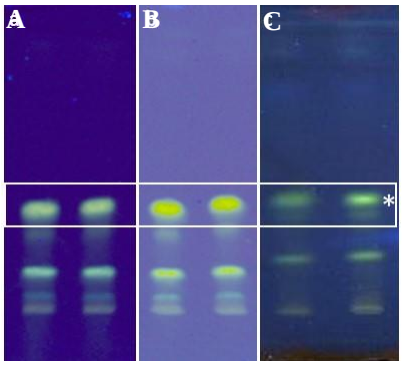

Figure 5. TLC precision of the *C. xanthorrhiza* extract. MP: CH_2Cl_2, $CHCl_3$, and C_2H_5OH (10: 10: 1). D: vanillin-sulfuric acid reagent, UV 366 nm. (*) marker compound

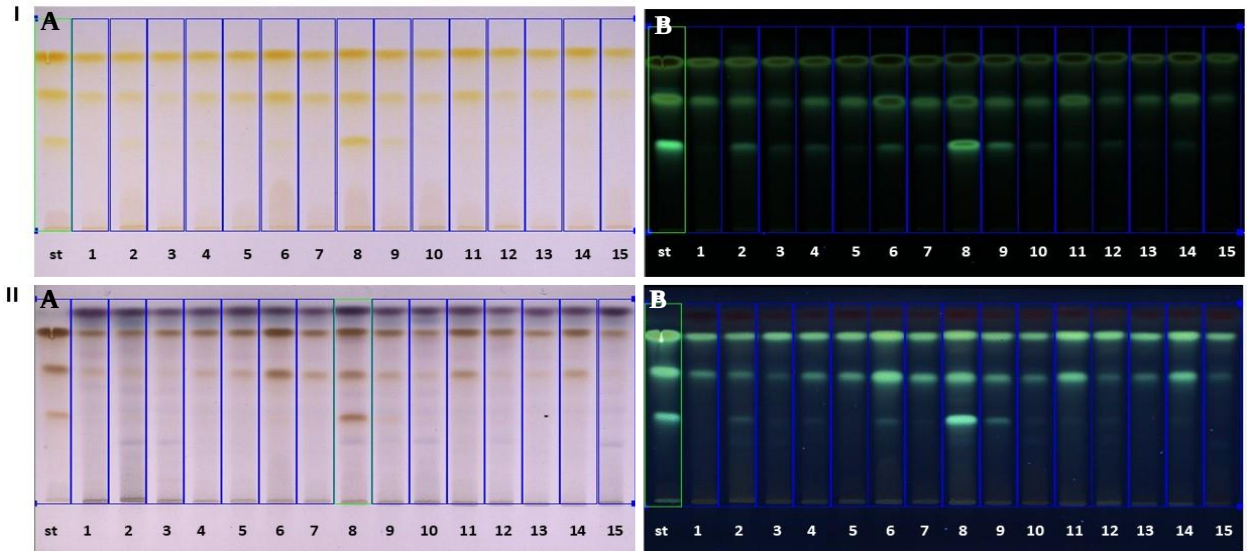

Figure 6. TLC-fingerprinting of *C. xanthorrhiza* from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15). MP: CH_2Cl_2, $CHCl_3$, and C_2H_5OH (10:10:1). D: before (**I**) and after (**II**) derivatization using a vanillin-sulfuric acid reagent, white light (**A**), UV 366 nm (**B**). St: standard of curcumin

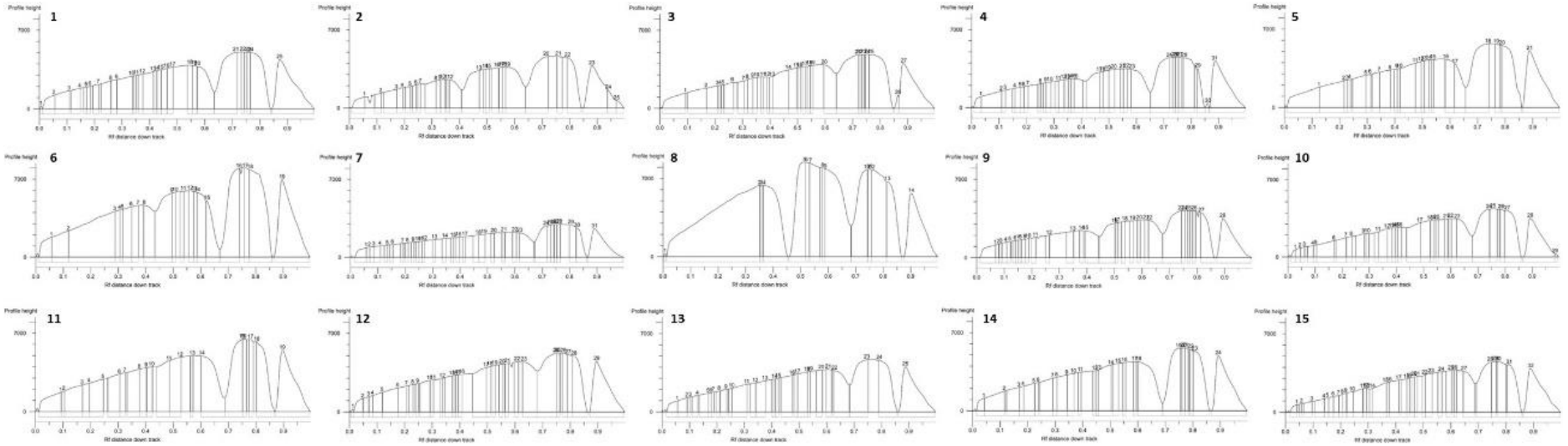

Figure 7. Video densitogram of *C. xanthorrhiza* from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15)

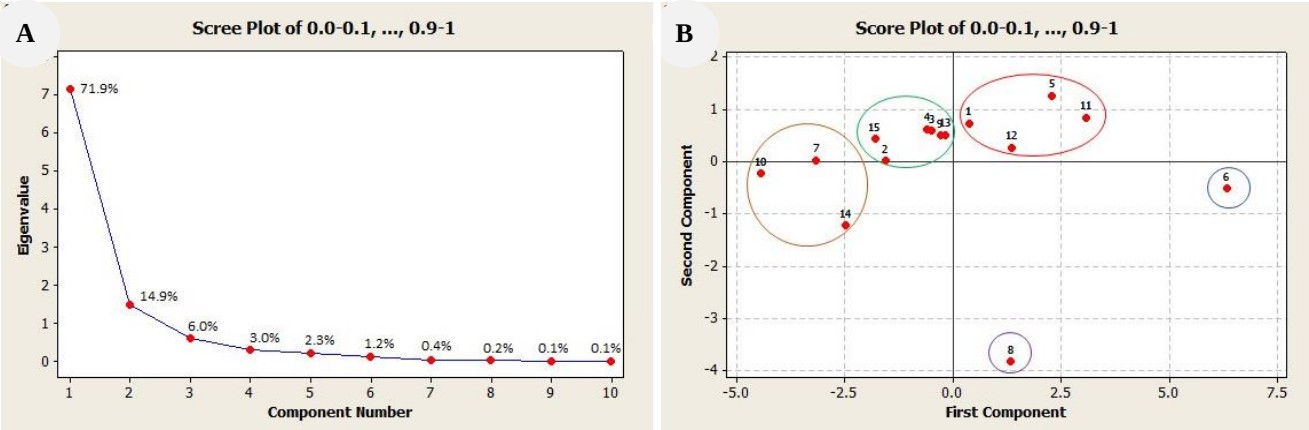

Figure 8. The scree plot of ten PCs from PCA (**A**) and the score plot of two PCs (PC1 dan PC2) from 15 samples of *C. xanthorrhiza* from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15) **(B)**

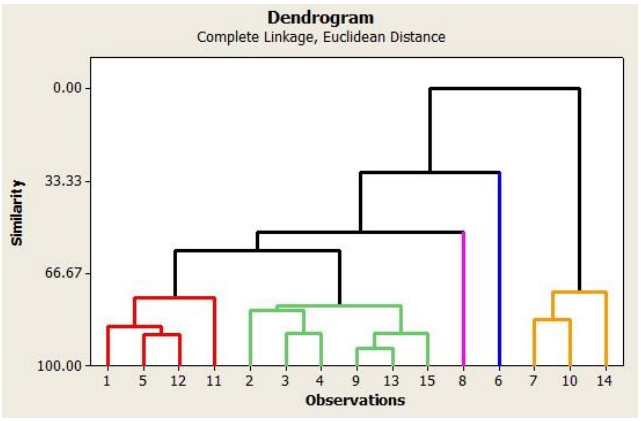

Figure 9. The dendrogram resulted from a complete linkage in the CA. 1-15 were *C. xanthorrhiza* from Tawangmangu (1), Batu (2), Sragen (3), Tulungagung (4), Bangkalan (5), Ngawi (6), Bojonegoro (7), Gresik (8), Pasuruan (9), Banyuwangi (10), Surabaya (11), Kediri (l2), Blitar (13), Palangkaraya (14), and Lombok Tengah (15)

Analysis using TLC followed by documentation using a TLC-visualizer on *C. xanthorrhiza* samples showed the presence of 21 components. Therefore, fingerprint analysis was then carried out based on these 21 compounds, using chemometric methods, namely PCA and CA. As seen from the PCA analysis results (Figure 8.A), the eigenvalues for principal components 1 and 2 (PC1 and PC2) and their cumulative were 71.9, 14.9, and 86.8%, respectively. These indicate that PC1 and PC2 explain 71.9 and 14.9% of the variability in the original observations, whereas both PCs accounted for 86.8% of the total variance. From the PC1 and PC2 score plots (Figure 8.B), the 15 *C. xanthorrhiza* rhizomes can be divided into five clusters: cluster 1 (Tawangmangu, Bangkalan, Kediri, and Surabaya), cluster 2 (Batu, Sragen, Tulungagung, Pasuruan, Blitar, and Lombok Tengah), cluster 3 (Ngawi), cluster 4 (Gresik), and cluster 5 (Bojonegoro, Banyuwangi, and Palangkaraya).

Cluster Analysis (CA) is a clustering method to ensure that similar samples are grouped in the same cluster. Each sample can form a cluster based on the distance between the cluster components; hence, the two closest points are combined to form a new cluster. The procedure is repeated continuously until all points are grouped, then the results are visualized in a dendrogram. The dendrogram presented in Figure 9 shows that the *C. xanthorrhiza* rhizomes collected from 15 Indonesian regions are divided into five clusters. Cluster 1 consists of samples from Tawangmangu, Bangkalan, Kediri, and Surabaya because they have similar chromatogram profiles, while cluster 2 consists of pure, dry rhizome samples from Batu, Sragen, Tulungagung, Pasuruan, Blitar, and Lombok Tengah. Clusters 3 and 4 comprise only one sample: *C. xanthorrhiza* rhizomes from Gresik and Ngawi, respectively. Lastly, cluster 5 consists of Bojonegoro, Banyuwangi, and Palangkaraya rhizomes. The sample groupings generated from CA correspond to those from PCA. The results of this research are in line with the results of previous research conducted on turmeric rhizomes. Fingerprints combined with PCA and CA were able to differentiate turmeric rhizomes originating from various locations. The results of grouping by PCA also agree with the sample grouping by CA (Kartini et al. 2021).

Several *C. xanthorrhiza* grouped into one cluster in this current research are likely to have similar chemical phytoconstituents both qualitatively and quantitatively, or in other words, they have comparable quality. On the contrary, different clusters indicate plants with different qualities. The 15 *C. xanthorrhiza* rhizomes observed in this work were mostly from lowlands to midlands, except those from Tawangmangu and Batu.

However, the five clusters resulting from the CA are not necessarily related to the altitude of the geographical origin. The results of this study are consistent with Royani et al. (2014), which concluded that the andrographolide content in *Andrographis paniculata* varies according to location and that there is no correlation between the altitude and their concentration (Royani et al. 2014). The

clustering of *C. xanthorrhiza* in this current research can be attributed to many variables related to the conditions of the growing site that cannot be controlled, such as soil type, environmental humidity, rainfall, sunlight intensity, and plant habitat. Other variables such as seedling properties, harvested age, cultivation process, nutrient soil availability, and plant protection affect the clustering (Rosidi 2020). Because of these findings, further research is needed to determine what factors contribute.

In conclusion, the TLC-derived fingerprint profiles of *Curcuma xanthorrhiza* rhizomes can be adequately visualized by factoring in the chromatographic conditions: silica gel 60 F_{254} plate as the stationary phase, the combination of CH_2Cl_2, $CHCl_3$, and C_2H_5OH with a ratio of 10:10:1 as the mobile phase, samples spotted in a volume of 6 µL, zone width of 7 mm, and derivatization with a vanillin-sulfuric acid reagent. Furthermore, based on chromatogram-based validation results, the rhizome extract is stable on the TLC plate and in the extract solution during the chromatography and 60 minutes after derivatization. Also, the extract samples meet the requirements for intraday precision. Chemometric analysis using PCA-CA grouping the samples into five clusters: cluster 1 (Tawangmangu, Bangkalan, Kediri and Surabaya), cluster 2 (Batu, Sragen, Tulungagung, Pasuruan, Blitar, and Lombok Tengah), cluster 3 (Ngawi), cluster 4 (Gresik), and cluster 5 (Bojonegoro, Banyuwangi, and Palangkaraya).

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