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Characterization of the Curcuminoids Fingerprint Profile in Curcuma and Zingiber Genera by TLC – Digital Image Analysis

Anisa Lailatusy Syarifah[1], Rurini Retnowati[1*], Hermin Sulistyarti[1]

[1]Chemistry Department, Faculty of Mathematics and Natural Sciences, University of Brawijaya, Jl. Veteran 65145 Malang, Indonesia

[*]Corresponding email: rretnowati@ub.ac.id

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ABSTRACT

Curcuma longa, *C. xanthorrhiza*, *C. heyneana*, and *Zingiber cassumunar* contain high curcuminoid and have relatively similar yellow color. Therefore, they are potentially adulterated and difficult to differentiate in the form of powder. Hence, it is necessary to characterize the fingerprints compound profile by a simple and rapid method. This research aims to determine fingerprint compound profile of curcuminoid using Thin Layer Chromatography (TLC) and digital image analysis. The result of the research identified that the fingerprint compound profile of curcuminoid on the four rhizomes was obtained by TLC method using silica gel 60 GF254 as the stationary phase, chloroform: dichloromethane: methanol (13:6:1) as the mobile phase, and observation under UV 254 nm light and citroborate reagent. Thereafter, the digital image analysis was carried out using Image J software according to the gray value and % of RGB (red-green-blue) value. Based on gray value and % of RGB, both Curcuma and Zingiber genera were differentiated through curcumin compound (R_f 0.63), demethoxycurcumin (R_f 0.34), bisdemethoxycurcumin (R_f 0.21). The profile of fingerprint compound on *Curcuma longa, C. xanthorrhiza, C. heyneana,* and *Zingiber cassumunar* was differentiated through R_f 0.26; R_f 0.17; and R_f 0.10.

Keywords: Curcuma, Zingiber, curcuminoid, thin layer chromatography, gray value, RGB value

INTRODUCTION

Rhizome *Curcuma longa (CL), C. xanthorrhiza (CX), C. heyneana (CH), and Zingiber cassumunar (ZC)* belong to Zingiberaceae family. In Indonesia, *CL*, *CX*, and *CH* are known as *kunyit, temulawak,* and *temugiring*, are rhizome species included in the Curcuma genus. Meanwhile, *ZC* which is known as bangle is a species of the Zingiber genus. The four rhizomes have a yellow color derived from the curcuminoid compound. The yellow similarity causes the four rhizomes is become potentially adulterated so that it is difficult to differentiate them from one another, especially sample in the form of powder. Therefore, information on the profile of the curcuminoid fingerprint compound from each species is highly necessary.

The fingerprint compound profile is the overall profile of the chemical compound identifying the characteristic of the chemical compound in the extract [1]. Characterization of fingerprint compound profile can be carried out by spectroscopic and chromatographic methods, including IR, HPLC, and LC-MS/MS. The method has a high sensitivity; nevertheless, it requires expensive instruments and cannot be conducted in a laboratory with simple equipment. TLC method has high validity, hence, it is still used in Indonesian Herbal

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Pharmacopeae Volume I to determine fingerprint compounds [2]. TLC method is a simple, inexpensive, accurate chromatography. Profile characterization of the fingerprint compound using TLC was carried out by comparing the chromatogram of several samples and standard compounds. In the Indonesian Herbal Pharmacopeae Volume 1, the fingerprint compound of *CL*, *CX*, *CH*, and *ZC* was determined with TLC. The fingerprints compound on *CL* and *CH* was determined using silica gel 60 GF254 as the stationary phase, chloroform: methanol (95:5) as the mobile phase, using UV light λ 366 nm, and curcumin compound at R_f 0.62-0.65 was obtained [2]. *CX* and *ZC* also contain curcuminoid [1,3]. However, in Indonesian Herbal Pharmacopeae Volume I, curcuminoid was not utilized as fingerprint compounds of those two rhizomes due to its low curcuminoid concentration. The fingerprint compound of *CX* and *ZC* was characterized using silica gel 60 GF254 nm as the stationary phase and toluene:ethyl acetate (93:7) as the mobile phase. With such separation condition, *CX* fingerprint compound was obtained, that was xantorizol at R_f 0.50, whereas the fingerprint compound of *ZC*, that was cineol at Rf 0.55 [2]. Hence, the TLC method based on the existing circumstance is required to characterize the fingerprint compound of those four rhizomes in the same batch.

The factors influencing TLC separation were a stationary phase, mobile phase, and sample concentration. The separation occurred due to the difference in the analyte compound affinity in two phases, namely the stationary phase and the mobile phase. For the similar mobile phase, the composition difference in the mobile phase affected the separation of the compound [4]. Several papers are reported, to separate curcuminoid in *CL* using TLC, one of which was a research carried out by Parasivam *et al.* who applied the chloroform: methanol (48:2) [5]. The separation results obtained three compounds, namely curcumin (CRM) at R_f 0.66, demethoxycurcumin (DMC) at R_f 0.48, and bisdemethoxycurcumin (BDMC) at R_f 0.27. Besides, the research of Revathy *et al*., concluded that the mobile phase of chloroform: methanol (19:1) was appropriate of increasing a separation of CRM, DMC, and BDMC compound, respectively, those three compounds were Rf0.75; 0.55, 0.27 [6]. Nevertheless, in the research of Rafi *et al*., an analysis of curcuminoid fingerprint compound in *CL*, *CX*, and *ZC* rhizomes was carried out using the mobile phase which was different from Revathy *et al.*, where chloroform:dichloromethane (32.5:67.5) [1,6]. Nonetheless, a distance of compound separation of CRM, DMC, and BDMC was nearer compared to separation using a mobile phase of chloroform: methanol (19:1). The result of that research was CRM compound with R_f 0.05; DMC with R_f 0.14; and BDMC with R_f 0.37. Hence, this research determined a mobile phase which was appropriate of increasing curcuminoid compound separation to utilize it as a fingerprint compound of *CL*, *CX*, *CH*, and *ZC*.

The difference of mobile phase composition utilized in TLC caused polarity alteration, to either increase the separation or cause no compound separation. The polarity of the solvent as the mobile phase was correlated to dielectric constant. Hence, the mobile phase determination to increase the separation is important in TLC method, particularly, TLC carried out for several samples in the same plate. Consequently, this research carried out a mobile phase determination according to the polarization, namely hexane, ethyl acetate, dichloromethane, chloroform, and methanol. In addition to the mobile phase, the TLC separation was also influenced by sample concentration. Higher concentration compound required stationary phase with bigger capacity. It was affected by the retardation factor of the mobile phase [3]. If the sample concentration is too high, it may cause the compound does not separate properly.

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TLC chromatograms of curcuminoid compounds can be visually observed using UV light (254 nm and 366 nm) and citroborate reagents [7]. These compounds were colorless at λ 254 nm, which were yellow when observed at λ 366 nm, and turned orange after being sprayed with citroborate reagent. Besides, if the rhizome extract concentration was low, the stains produced on the plate was not apparent. Consequently, the chromatogram documentation with a digital camera created a digital image which was hard to observe. However, it could be overcome by combining TLC and analysis of digital images. Digital image analysis was conducted with Image-J software, based on the gray value [8] and % RGB value [9]. The gray value parameter illustrated the value of gray intensity, while the % RGB value indicated the intensity of red, green, and blue. The gray value was evaluated to determine the TLC separation pattern carried out so that a proper mobile phase can be set out. The percentage of RGB values was applied to decide the intensity of each compound as a result of the TLC separation.

Hence, this research was carried out to characterize curcuminoid fingerprint compound of Curcuma and Zingiber genera by a fast and simple method, namely the TLC method and digital image analysis with Image J software [10]. A various mobile phase and extract concentration were carried out to characterize the separation circumstance utilizing TLC method to separate compounds in methanol extract and characterize curcuminoid fingerprint compound profile in *CL, CX, CH,* and *ZC*. Visualization of fingerprint compound profile which can differentiate *CL, CX, CH,* and *ZC* was selected based on a grey value graph dan % RGB.

EXPERIMENTAL

Chemicals

Standard of curcumin (CRM), desmethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC) were purchased from Sigma Aldrich (St. Louis, USA). Silica gel 60 GF_{254} TLC plates (Macherey-Nagel, Germany). Hexane, dichloromethane, ethyl acetate, chloroform, methanol, citric acid, and boric acid employed in this research had analyst grade which was obtained from Merck.

Plants material

Fresh rhizomes of *C. longa (CL), C. xanthorrhiza (CX), C. heyneana (CH),* and *Z. cassumunar (ZC)* rhizomes were obtained and identified at UPT Materia Medica, Batu, East Java.

Preparation of standard and samples solutions

The sample solution was produced through these following steps: the fresh rhizomes samples were washed, sliced (size 3-6 mm), dried using an oven at a temperature of 45 ^{o}C. Then, it was pulverized so it became simplicia powder. The simplicia powder was sieved using 40-100 mesh sieves. Simplicia powder was macerated with methanol for 1 hour by stirring with a stirrer. The process was carried out on two variations of the powder (weight) and methanol solvents (volume). The ratio of 1 was formulated by comparison (1:200), so that the methanol extract *CL-1*, *CX-1*, *CH-1*, and *ZC-1* was obtained. The ratio of 2 was formulated by comparison (1:10), so that the methanol extract *CL-2*, *CX-2*, *CH-2*, and *ZC-2* was acquired. Furthermore, each methanol extract was tested for the presence of curcuminoid compound using citroborate reagent. Standard solutions of CRM, DMC, and BDMC were dissolved in methanol at a concentration of 0.01 % g/mL. The eight methanol extracts and three standard solutions were stored in the refrigerator at 4 ^{o}C.

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Chromatographic conditions

The standard solutions (CRM, DMC, BDMC) (4 µL) and methanol extracts (CL, CX, CH, ZC) (12 µL) were applied to silica gel 60 GF_{254} plate (8 x 8 cm) as the stationary phase. The stationary phase was developed in the twin bottom chamber previously saturated with the mobile phase for 15 minutes. Various mobile phase used are methanol, dichloromethane, chloroform, ethyl acetate, n-hexane, hexane:ethyl acetate (5:5, 6:4, 8:2 v/v); chloroform: dichloromethane (5:5, 3:7, 2:8 v/v); chloroform:methanol (10:10, 16:4, 18:2, 19:1 v/v); chloroform:methanol:dichloromethane (12:6:2 and 13:6:1 v/v/v). After elution, the plate was removed and air-dried. Then, chromatogram was observed using UV light at λ 254 and 366 nm, without UV light and citroborate reagents, then each separate stain was determined the value of the R_f, and the digital image was analyzed using Image J software [11]. The value of R_f stain resulted from the separation was compared with R_f values of the CRM, DMC, and BDMC of the standard compounds.

Digital Image Analysis Using Image J Software

Each TLC chromatogram obtained from the chromatography procedure above was inserted into the UV lamp and cabinet (CAMAG) and observed with UV light (254 and 366 nm). Each observation utilizing UV light was photographed with a digital camera with 13 megapixel resolution and each image was transferred by the scanner. The digital images results were stored in computer data in the format of a joint photographic expert group (JPEG) files. Those digital images were analyzed by gray value and % RGB using free Image J 1.50i software (Wayne Rasband, National Institutes of Health, USA). It comprised the graph of the gray value relationship with distance (pixel) and the average digital value of RGB (Red, Green, Blue). The gray value (x, y) graph consisted of the x-axis (distance) and the y-axis (gray value). Distance (pixel) was altered in a cm scale. Before determining the gray value graph, digital images were converted in 16-bit format [11]. The percentage of each R, G, and B value was defined using the formula %R = ((mean R / (mean R + mean G + mean B)) x 100%. The percentage of G and% B was formulated in the same method.

RESULT AND DISCUSSION

In this research, the determination of the curcuminoid fingerprint compounds profile in Curcuma and Zingiber genera was carried out with TLC method using curcumin (CRM), demetoxicurcumin (DMC) and bisdemetoxicurcumin (BDMC) standard. The profile of standard compounds of CRM, DMC, and BDMC, was determined using silica gel GF_{254} as the stationary phase and several varied mobile phases. The composition variations of the mobile phase consisted of hexane, ethyl acetate, methanol, dichloromethane, chloroform, and chloroform: methanol. The value of R_f of each standard compound of CRM, DMC and BDMC respectively is shown on the Table 1.

According to the data in the Table 1, the mobile phase of hexane was not able to elute the compounds of CRM, DMC, and BDMC standard. The use of very polar mobile phase such as ethyl acetate and methanol showed that CRM, DMC, and BDMC standard compounds was able to be eluted and obtained one stain respectively. The use of chloroform as the mobile phase, showed that each standard had different R_f values, DMC (R_f 0.18) and BDMC (R_f 0.05). It showed that the two compounds had different affinity to the mobile phase. Each of the compounds would be carried by mobile phase or would be more retained due to the stationary phase depending on the affinity of the compounds to the mobile phase and stationary phase.

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The eluent polarity was sequenced as follows: hexane – chloroform – dichloromethane – ethyl acetate – methanol [3].

The profile of the standard compound of CRM had been determined by TLC method using chloroform as the mobile phase and observed under UV 366 nm light, was obtained three yellow stains, stain 1 (R_f 0.35), stain 2 (R_f 0.18), and stain 3 (R_f 0.05). The stain 2 had the similar R_f value as DMC standard and the stain 3 had the similar R_f value as BDMC standard. The profile of CRM standard compound determined using chloroform: methanol (19:1) as the mobile phase obtained three yellow stains. They were stain 1 (R_f 0.65), stain 2 (R_f 0.38), and stain 3 (R_f 0.24). The stain 2 had the similar R_f value as DMC standard and the stain 3 had the same R_f value as BDMC standard. Therefore, the stain 2 and the stain 3 respectively were assumed as DMC and BDMC compounds. In addition, the stain 1 (R_f 0.65) was assumed as CRM compound in accordance with the Indonesian Herbal Pharmacopeia Volume 1 [2]. The determination of CRM fingerprints compounds on *CL* and *CH* by TLC using silica gel 60 GF_{254} as the stationary phase and chloroform: methanol (95:5) as the mobile phase, λ 366 nm UV light stain visualizer showed that R_f value of CRM was 0.62-0.65 [2]. In conclusion, the descriptions above showed that CRM standard compound could be separated from its mixture compounds, DMC and BDMC, using chloroform: methanol (19:1) as the mobile phase.

Table 1. The R_f values of each standard compounds

Mobile phase	Standard					
	CRM		DMC		BDMC	
	Σ	R_f values	Σ	R_f values	Σ	R_f values
Hexane	0	-	0	-	0	-
Chloroform	3	0.35 0.18 0.05	1	0.18	1	0.05
Dichloromethane	2	0.35 0.07	1	0.07	0	0
Ethyl acetate	1	0.85	1	0.85	1	0.85
Methanol	1	0.82	1	0.82	1	0.82
Chloroform : methanol (19:1)	3	0.65 0.38 0.24	1	0.38	1	0.24

Descriptions: Σ is amount of stain; CRM (curcumin); DMC (demethoxycurcumin); BDMC (demethoxycurcumin)

The profile of fingerprint compound was the profile of chemical compounds in *C. longa* (*CL*), *C. xanthorrhiza* (*CX*), *C. heyneana* (*CH*), and *Z. cassumunar* (*ZC*) methanol. The determination of the fingerprint compound profile of curcuminoid of *CL*, *CX*, *CH*, and *ZC* extract was carried out with TLC method using CRM, DMC, and BDMC standards. The TLC method was carried out using silica gel of 60 GF_{254} as the stationary phase and several varied mobile phases. The variation of the mobile phases consisted of two and three types of eluent, which were n-hexane: ethyl acetate, chloroform: dichloromethane, chloroform: methanol, and chloroform: dichloromethane: methanol. The observation of TLC was carried out under UV 254 and 360 nm light and citroborate reagent. The observation under UV 254 nm light was used to observe compound which was not able to absorb UV 366 nm light. Citroborate reagent was used to identify curcuminoid compounds looked yellow in color when being observed under UV 366 nm light and color change occurred becoming reddish orange when it reacted to

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citroborate reagent. According to Sirait *et al.*, the color change is due to formation of the complex compounds occurring because of reaction between boron and hydroxyl and carbonyl groups on curcuminoid [7]. Structure of the complex compounds between curcumin and boric acid consisted of two forms, rubrocurcumin and rososianin. Besides, according to Priyadarsini *et al.* [12], curcumin will be red in color when its pH is <7.

Table 2. R_f values of each stain on the TLC chromatogram of *CL-1*, *CX-1*, *CH-1*, and *ZC-1* methanol extracts

Mobile phase	Standard and extract													
	CRM		DMC		BDMC		CL-1		CX-1		CH-1		ZC-1	
	Σ	R_f values	Σ	R_f values	Σ	R_f values	Σ	R_f values	Σ	R_f values	Σ	R_f values	Σ	R_f values
HE : EA (5:5)	1	0.32	1	0.34	1	0.32	1	0.35	1	0.34	1	0.34	1	0.31
HE : EA (6:4)	1	0.28	1	0.28	1	0.28	1	0.29	1	0.28	1	0.28	1	0.28
HE : EA (8:2)	1	0.015	1	0.015	1	0.015	1	0.015	1	0.015	1	0.015	1	0.015
KL : DM (2:8)	2	0.15 0.046	1	0.046	1	0	2	0.15 0.046	2	0.15 0.046	2	0.15 0.046	1	0.14
KL : DM (3:7)	2	0.15 0.046	1	0.046	1	0	2	0.15 0.046	2	0.15 0.046	2	0.15 0.046	1	0.14
KL : DM (5:5)	2	0.15 0.046	1	0.046	1	0	2	0.15 0.046	2	0.15 0.046	2	0.15 0.046	1	0.14
KL : ME (10:10)	1	0.89	1	0.89	1	0.89	1	0.89	1	0.89	1	0.89	1	0.89
KL : ME (16:4)	1	0.74	1	0.69	1	0.66	1	0.69	1	0.69	1	0.69	1	0.74
KL : ME (18:2)	1	0.80 0.69 0.58	1	0.69	1	0.58	3	0.80 0.69 0.58	3	0.80 0.69 0.58	3	0.80 0.69 0.58	1	0.80
KL : ME (19:1)	3	0.65 0.38 0.24	1	0.38	1	0.24	5	0.62 0.46 0.34 0.25 0.20	4	0.62 0.46 0.34 0.20	4	0.62 0.46 0.34 0.20	1	0.62
KL:DM:ME (12 : 6 : 2)	3	0.85 0.75 0.65	1	0.75	1	0.65	3	0.85 0.75 0.65	3	0.85 0.75 0.65	3	0.85 0.75 0.65	1	0.85
KL:DM:ME (13 : 6 : 1)	3	0.65 0.37 0.21	1	0.48	1	0.34	5	0.63 0.44 0.34 0.26 0.21	4	0.63 0.44 0.34 0.21	4	0.63 0.44 0.34 0.21	1	0.63

Descriptions: Σ is amount of stain, CRM (curcumin); DMC (demethoxycurcumin); BDMC (demethoxycurcumin); CL (*C. longa*); CX (*C. xanthorrhiza*); CH (*C. heyneana*); ZC (*Z. cassumunar*).

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The R_f values of each compound on TLC chromatogram of *CL-1*, *CX-1*, *CH-1*, and *ZC-1* extract is shown in Table 2. Based on the data in the Table 2, considering the amount of stains and separation distance of each stain, TLC used chloroform : methanol (19:1) and chloroform: dichloromethane: methanol (13:6:1) as the mobile phase which was able to separate compound in *CL*, *CX*, *CH*, and *ZC* methanol extracts better than other compositions. Using chloroform: methanol (19:1) as the mobile phase and observation under UV 366 nm light, 5 stains (as shown on the Figure 1) were obtained. Three stains had the same R_f value as the compound standard of CRM, DMC, and BDMC, which were $R_f(2)$ 0.62; $R_f(4)$ 0.34; $R_f(6)$ 0.20 respectively. Moreover, TLC using chloroform: methanol (19:1) as the mobile phase was able to separate CRM standard compound from its mixture compounds, which were DMC (R_f 0.38) and BDMC (R_f 0.24) compounds. Observation under UV 254 nm light obtained 7 stains in *CL-1* methanol extract, however the separation distance of stains which was on $R_f(5)$ – $R_f(8)$ was regarded too close, R_f 0.25; 0.20; 0.14; and 0.09 respectively. Therefore, in order to increase the compound separation in the extract, mobile phase composition consisting of three types of eluent, which were chloroform: dichloromethane: methanol, was used.

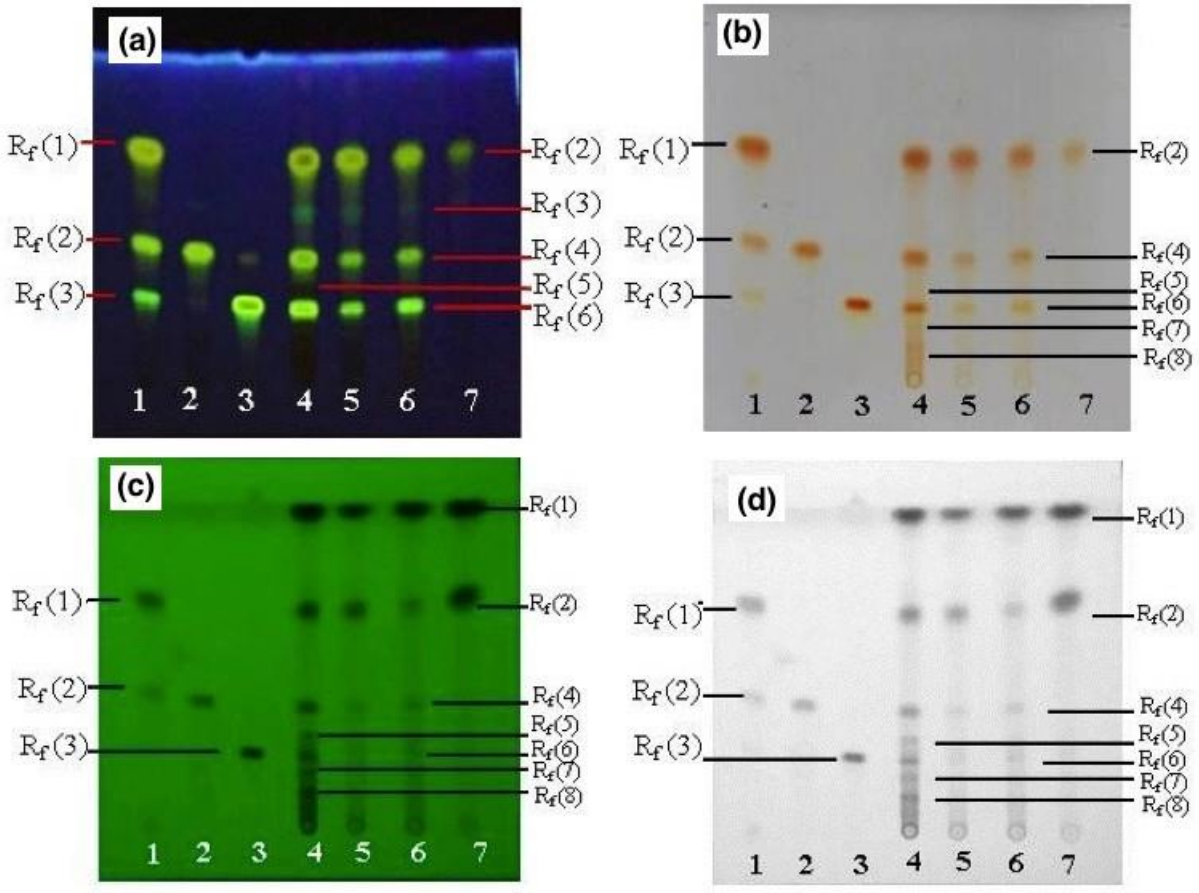

Figure 1. TLC chromatogram of standard compounds and methanol extracts from TLC using chloroform: methanol (19 : 1, v/v) as the mobile phase which was observed under: (a) : UV 366 nm; (b): citroborate reagent; (c) : UV 254 nm; (d) : Digital image of TLC chromatogram (number c) after being converted into 16 bit type. Descriptions: 1-3 = CRM, DMC, and BDMC standard, 4 – 7 = *CL-1*, *CX-1*, *CH-1*, *ZC-1* methanol extracts.

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According to the research, chloroform: dichloromethane: methanol (13:6:1) used as the mobile phase were able to increase the separation of compounds in *CL*, *CX*, *CH*, and *ZC* methanol extracts compared to the use of chloroform: dichloromethane: methanol (12:6:2) as the mobile phase because 7 stains were obtained on the observation under UV 254 nm. The three stains had the similar R_f value as the standard compounds of CRM, DMC, and BDMC, which were R_f(2) 0.65; R_f(4) 0.37; and R_f(6) 0.21 respectively. The TLC chromatogram of the methanol extracts (*CL*, *CX*, *CH*, and *ZC)* from TLC using chloroform: dichloromethane: methanol (13:6:1) as the mobile phase was shown on the Figure 2. The R_f values of each stain on the TLC chromatogram using chloroform : methanol as the mobile phase was shown in Table 3.

Table 3. R_f values of standard compounds and methanol extracts on TLC chromatogram from TLC using chloroform: dichloromethane: methanol (13:6:1) as the mobile phase

Observation methods	R_f values						
	CRM	**DMC**	**BDMC**	**CL**	**CX**	**CH**	**ZC**
(a) with UV 254 nm	0.65 0.37 0.21	0.37	0.21	0.92 0.63 - 0.34 0.26 0.21 0.17 0.10	0.92 0.63 - 0.34 - 0.21	0.92 0.63 - 0.34 - 0.21	0.92 0.63
(b) with UV 366 nm	0.65 0.37 0.21	0.37	0.21	- 0.63 0.44 0.34 0.26 0.21 - -	- 0.63 0.43 0.34 - 0.21	- 0.63 0.43 0.34 - 0.21	- 0.63
(c) with citroborate reagent	0.65 0.37 0.21	0.37	0.21	- 0.63 - 0.34 0.26 0.21 0.17 0.10	- 0.63 - 0.34 - 0.21	- 0.63 - 0.34 - 0.21	- 0.63

Description: The observation use (a) UV 254 nm, (b) UV 366 nm; (c) citroborate reagent and (-) stain is not detected.

The separation result using chloroform: dichloromethane: methanol (13:6:1) as the mobile phase (Figure 2) was almost similar with the result of separation using chloroform : methanol (19:1) (Figure 1) as the mobile phase. However, the result of separation using chloroform: dichloromethane: methanol (13:6:1) as the mobile phase was better than the use

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of chloroform : methanol (19:1) as the mobile phase. The difference could be seen from the chromatogram of *CL-1* extract observed under UV 254 nm. The difference of the separation pattern obtained from both of the compositions of the mobile phase above was then analyzed based on gray value graph of *CL-1* extract using Image J software shown on the Figure 3.

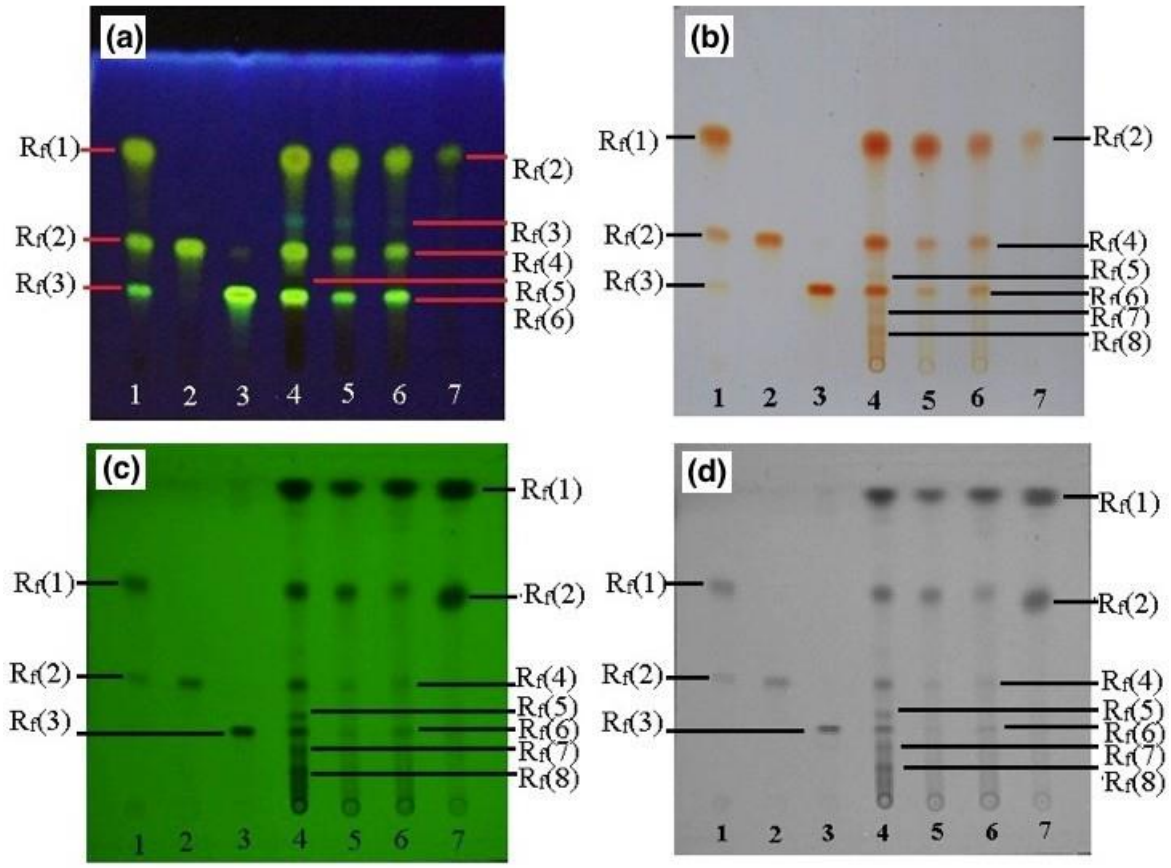

Figure 2. TLC chromatogram of standard compounds and methanol extracts from TLC using chloroform: dichloromethane: methanol (13:6:1, v/v) as the mobile phase which was observed under: (a) : UV 366 nm; (b): citroborate reagent; (c) : UV 254 nm; (d) : Digital image of TLC (number c) after being converted into 16 bit type. Descriptions: 1 - 3 = CRM, DMC, and BDMC standard; 4 – 7 = *CL-1*, *CX-1*, *CH-1*, *ZC-1* methanol extracts.

In this study, x axis on the gray value graph (Figure 3) indicates the distance from sample point to the limit of the mobile phase elution and y axis indicates intensity. Every peak of gray value shows color intensity of each stain. The gray value graph on Figure 3 is the graph of grayish color intensity of chromatogram TLC, an observation result on λ 254 nm (Figure 1.c and 2.c). The higher intensity approaching value of 255 shows the domination of gray-white color. On the other hand, the lower intensity approaching value of 0 shows the domination of blackish gray color.

According to the gray value graph (Figure 3), chloroform: dichloromethane: methanol (13:6:1) used as the mobile phase are able to increase the compound separation on R_f (5), R_f (6), and R_f (7) respectively than chloroform: methanol (19:1). It can be seen on the gray value graph in which the peak of R_f (5) which is red in color is looked sharper. The peak indicates the limit of difference of gray and white colors intensity, while the peak of R_f (5) on the gray

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value graph which is black in color is more sloping therefore the limit of the intensity difference between the black and gray colors can't be obviously seen.

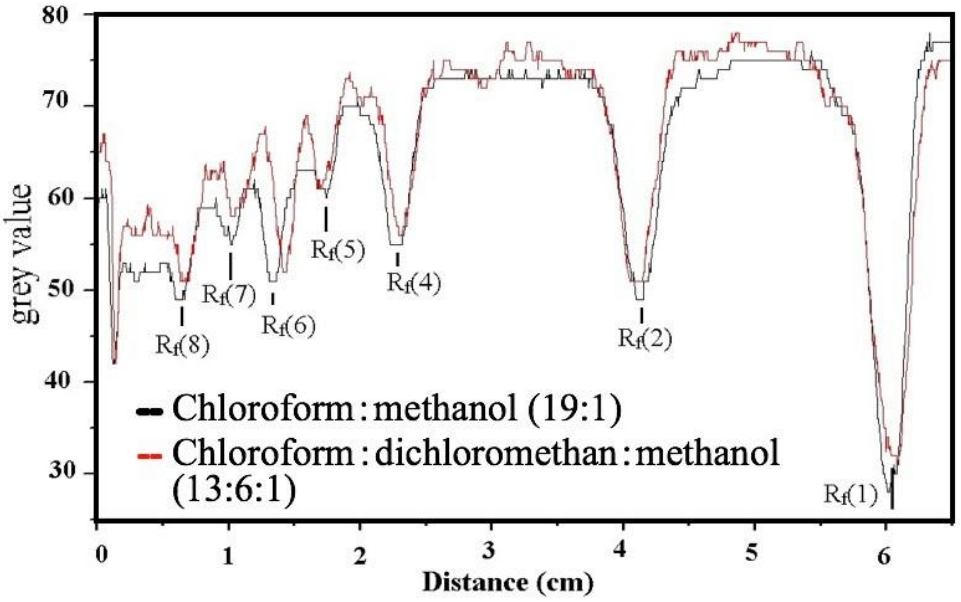

Figure 3. Gray value graph from the digital image of TLC chromatogram of *CL-1* methanol extract observed under UV 254 nm and after being converted into 16 bit type. Descriptions: 1-3 = CRM, DMC, and BDMC standard, and 4 – 7 = *CL*, *CX*, *CH*, *ZC* methanol extracts.

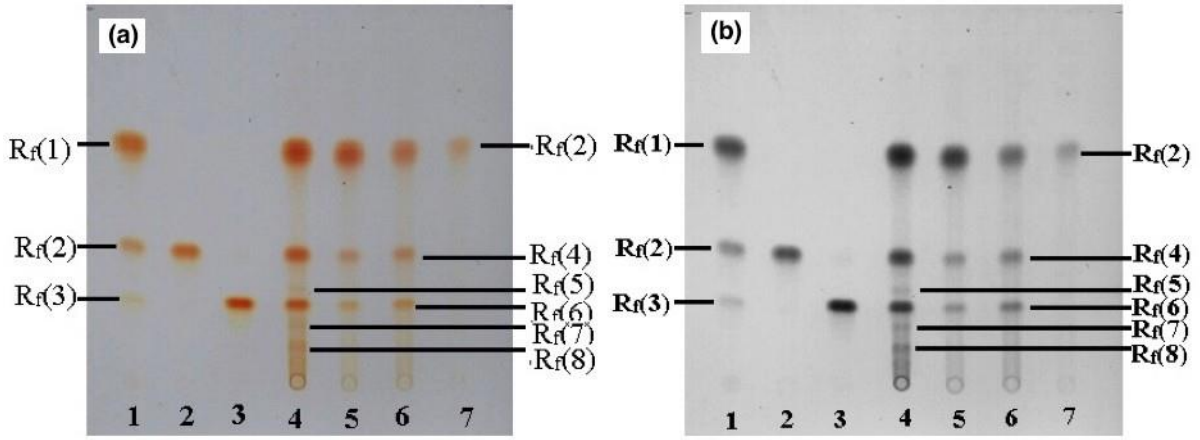

Figure 4. (a) TLC chromatogram of *CL-1* methanol extract (rasio 1:200) from TLC using chloroform:dichloromethane:methanol (13:6:1) as the mobile phase and observed under citroborate reagent, (b) after being converted into 16 bit type. Descriptions: (a, b) 1 - 3 = CRM, DMC, and BDMC standard; 4 – 7 = *CL-1*, *CX-1*, *CH-1*, and *ZC-1* methanol extracts.

The extract concentration was able to influence separation, in which compound having higher concentration needed mobile phase with bigger capacity. The influence of the extract concentration could be analyzed using gray value graph shown on Figure 5 and 7. Based on the gray value graph, the increase of the extract concentration from ratio of 1:200 (Figure 4) to 1:10 (Figure 6), caused some compounds of (R_f (7) and R_f (8) becoming more difficult to

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separate because of the occurrence of tailing. Due to the fact, the determination of the fingerprint compound used extract concentration with 1:200 ratios.

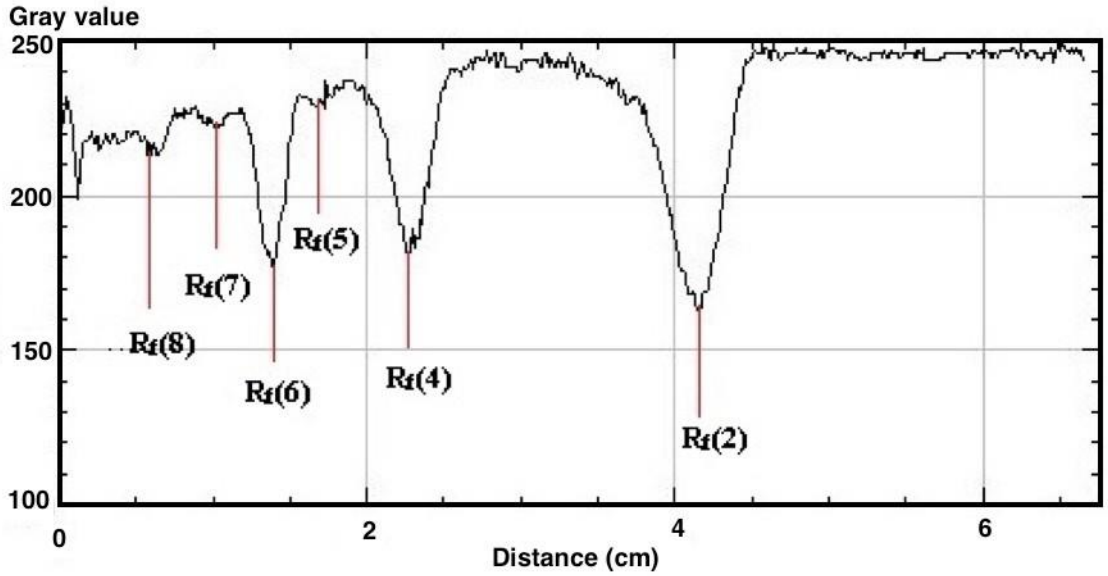

Figure 5. Gray value graph from the digital image of TLC of *CL-1* methanol extract and after being converted into 16 bit type.

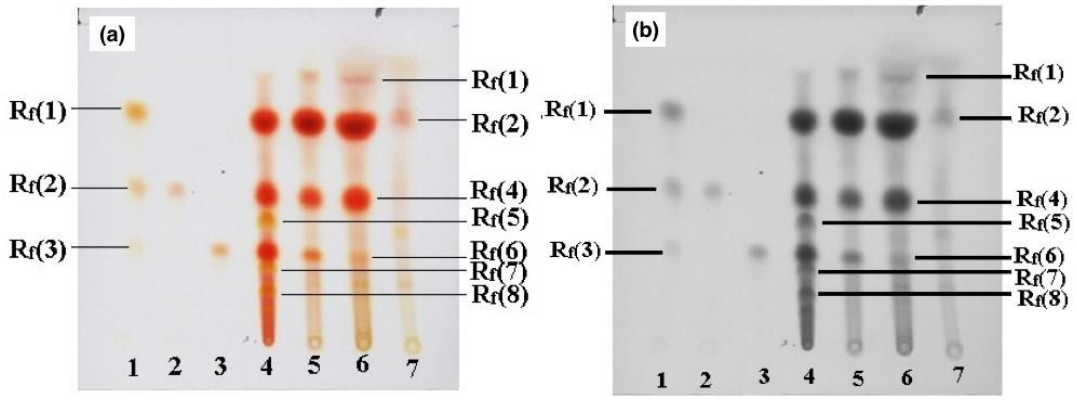

Figure 6. (a) TLC chromatogram of *CL-2* methanol extract (rasio 1:10) from TLC using chloroform: dichloromethane: methanol (13:6:1) as the mobile phase and observed under citroborate reagent, (b) after being converted into 16 bit type. Descriptions: (a,b) 1 - 3 = CRM, DMC, and BDMC standard; 4 – 7 = *CL-2*, *CX-2*, *CH-2*, and *ZC-2* methanol extracts

According to the previous descriptions, the TLC condition suitable to characterize curcuminoid profile on Curcuma and Zingiber genera was TLC using silica gel 60 GF_{254} as the stationary phase, and chloroform: dichloromethane: methanol (13:6:1) as the mobile phase, UV (254 and 366 nm) light and citroborate reagent as the stain visualizer. With such condition of TLC, in accordance with Figure 2, curcuminoid color is greenish when being observed under UV 366 nm light and it becomes red-orange when reacting with citroborate reagent. According to data in Table 3, *CL*, *CX*, *CH* methanol extract can be differentiated from *ZC*, in which 3

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typical stains with R_f(2) 0.63; R_f(4) 0.34; and R_f(6) 0.21 respectively are obtained. The three stains have the same R_f as the standard of CRM, DMC, and BDMC. While for the genus of Zingiber, *ZC* methanol extract only obtained 1 typical stain with R_f 0.63 which has the same R_f as CRM standard. The characterization of fingerprint compound profile of *CL*, *CX*, and *CH* is easier to observe under UV 254 nm light and citroborate reagent before being analyzed with Image J software (gray value graph and % *RGB*). Figure 8.A is the gray value graph of TLC digital image of *CL-1*, *CX-1*, *CH-1*, and *ZC-1* (observation result under UV 254 nm). On the gray value graph of *CL-1* 7 peaks are obtained and three peaks, R_f(5) 0.26; R_f(7) 0.17; R_f(8) 0.10, are only detected under *CL* methanol while ZC methanol extract has only two peaks.

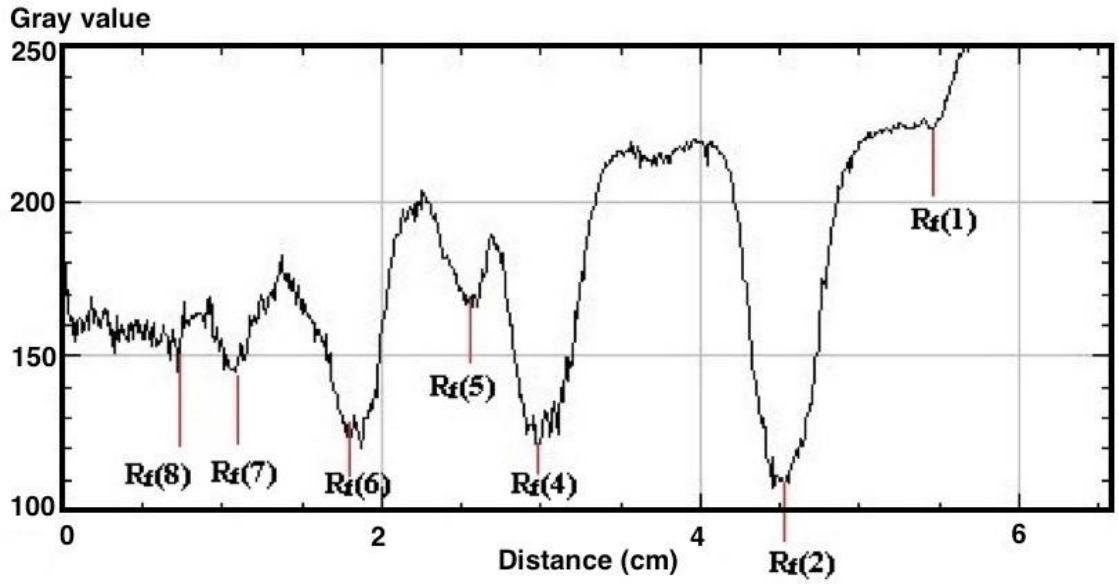

Figure 7. Gray value graph from the digital image of TLC of *CL-2* methanol extract and after being converted into 16 bit type.

Figure 8.B is the gray value graph of *CL-1*, *CX-1*, *CH-1*, and *ZC-1* TLC digital image (observation result under citroborate reagent). The change of red-orange colors occurred on curcuminoid compound reacting with citroborate reagent. The higher the curcuminoid compound content reacting with citroborate reagent, the higher the color intensity. According to graph in Figure 8.B, *CL*, *CX*, and *CH* can be differentiated based on the peak intensity of R_f(4) and R_f(6) which are respectively assumed as the peaks of DMC and BDMC compounds. The color intensity is also indicated by the % *RGB (red-green-blue)*. The value of *RGB* of *CL*, *CX*, *CH*, and *ZC* methanol extracts is shown in Table 4. The data of the *RGB* value was obtained from the *RGB* values average of each stain observed under citroborate reagent. The difference of % *RGB* can be detected from the orange intensity obtained. The difference of the orange intensity could be detected from the percentage amount of its complementary color, the blue color (% B). The stronger the orange intensity, the lower the % B value. Therefore according to gray value graph in Figure 8.B. and Table 4 it can be assumed that the concentration of CRM, DMC, and BDMC compounds in *CL* methanol extract is higher than *CX*, *CH*, and *ZC* methanol extracts. The concentration of DMC and BDMC compounds in the *CX* and *CH* methanol extracts is equal, but the *CH* methanol extract has higher concentration of DMC and BDMC compound than in *CX* methanol extract. On the other hand, the concentration of CRM

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compound in *ZC* methanol extract is constantly lower than in *CH*, *CX*, and *CL* methanol extracts.

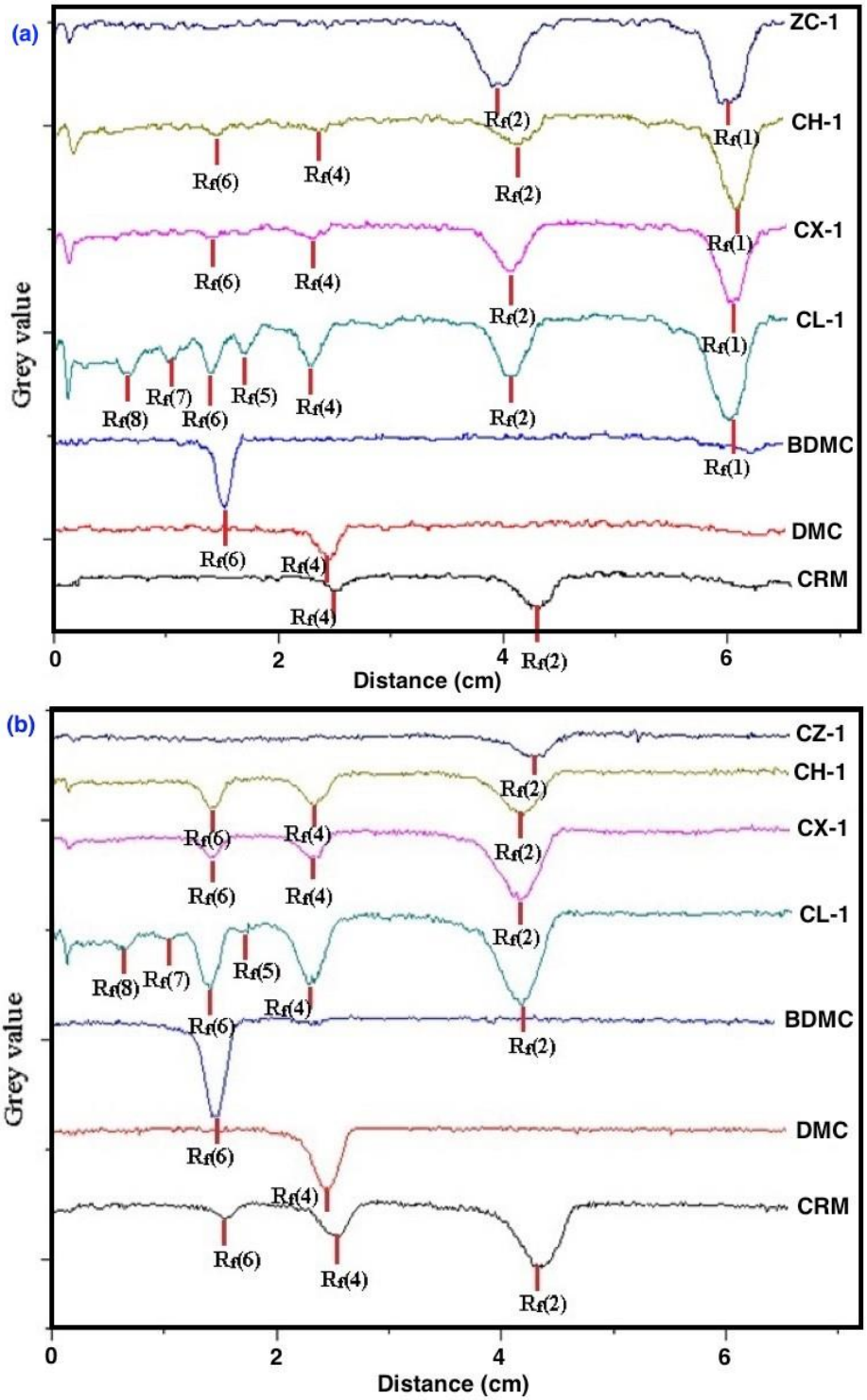

Figure 8. Gray value graph of standard solution (CRM, DMC, BDMC) and methanol extracts (*CL-1*, *CX-1*, *CH-1*, and *ZC-1*). The TLC operational condition: solvent used as a mixture of chloroform: dichloromethane: methanol with ratio (13: 6:1). Observation under UV light at 254 nm (a) and citroborate reagent (b).

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Table 4. RGB values of each compound on the TLC chromatogram of *CL-1*, *CX-1*, *CH-1*, and *ZC-1* methanol extract after observed under citroborate reagent (Figure 4.a)

Extract	RGB values					
	R_f (2) 0.63	R_f (4) 0.34	R_f (5) 0.26	R_f (6) 0.21	R_f (7) 0.17	R_f (8) 0.10
Methanol extract CL-1						
Red (R)	246	248	245	248	243	243
Green (G)	177	188	233	183	225	217
Blue (B)	133	149	212	134	200	184
%R	44	42	36	44	36	38
%G	32	32	34	32	34	34
%B	24	25	31	24	30	29
Methanol extract CX-1						
Red (R)	245	246	-	245	-	-
Green (G)	187	221	-	226	-	-
Blue (B)	156	203	-	198	-	-
%R	42	37	-	37	-	-
%G	32	33	-	34	-	-
%B	27	30	-	30	-	-
Methanol extract CH-1						
Red (R)	245	245	-	245	-	-
Green (G)	206	215	-	216	-	-
Blue (B)	183	191	-	179	-	-
%R	39	38	-	38	-	-
%G	32	33	-	34	-	-
%B	29	29	-	28	-	-
Methanol extract ZC-1						
Red (R)	245	-	-	-	-	-
Green (G)	226	-	-	-	-	-
Blue (B)	207	-	-	-	-	-
%R	36	-	-	-	-	-
%G	33	-	-	-	-	-
%B	31	-	-	-	-	-

Descriptions: (-) : stain is not detected

CONCLUSION

According the result of the research, it can be concluded that the characterization of curcuminoid fingerprint compound of Curcuma and Zingiber genera could be carried out by TLC method-digital image analysis using Image J software. The appropriate TLC circumstance to differentiate CL, CX, CH, and ZC was by using silica gel 60 GF_{254} as stationary phase, chloroform: dichloromethane: methanol (13:6:1) as mobile phase, observation under UV 254 nm, and citroborate reagent. According to the gray value and % *RGB* value, the Curcuma and Zingiber genera could be differentiated through curcumin (R_f 0.63), demethoxycurcumin (R_f 0.34), bisdemethoxycurcumin (R_f 0.21) compounds. The profile of fingerprints compounds on

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Curcuma longa, C. xanthorrhiza, C. heyneana, and *Zingiber cassumunar* were differentiated through some compounds of R_f 0.26; R_f 0.17; and R_f 0.10.

CONFLICT OF INTEREST

Authors declare no competing interest published the manuscript.

REFERENCES

[1] Rafi, M., Rohaeti, E., Miftahudin, A., Darusman, L.K., *Indones. J. Chem.*, **2011**, 11 (1), 71–74
[2] Departemen Kesehatan Republik Indonesia, Farmakope Herbal Indonesia Edisi I, **2008**, Departemen Kesehatan Republik Indonesia, Jakarta
[3] Nihayati, Ellis, Tatik Wardiyati, Rurini Retnowati, and Soemarno, *Agrivita J. Agri. Sci.*, **2013**, 35 (3), 218-226.
[4] Wonorahardjo, Surjani, Metode-Metode Pemisahan Kimia, **2016**, Indeks Akademia, Jakarta
[5] Paramasivam, M., Poi, R., Banerjee, H., Bandyopadhyay, A., *Food Chem*., **2009**, 113 (2), 640–644.
[6] Revathy, S., S. Elumalai, Merina Benny, and Benny Antony, *J. Exp. Sci.*, **2011,** 2(7), 21-25.
[7] Sirait, Midian, Penuntun Fitokimia dalam Farmasi, **2007**, Penerbit ITB, Bandung.
[8] Tie-xin, Tang and Wu Hong, An Image Analysis System for Thin-Layer Chromatography Quantification and Its Validation, *J. Chromatogr. Sci.*, **2008**, 46 (6), 560-564.
[9] Firdaus, M. Lutfi, Wiwit Alwi, Ferli Trinoveldi, Iman Rahayu, Lena Rahmidar, Kancono Warsito, *Procedia Environ. Sci.*, **2014**, 20, 298-304.
[10] Rueden, C. T., Schindelin, J., Hiner, M.C., Dezonia, B.E., Walter, A.E., Arena, E.T. and Eliceiri, K.W., *BMC Bioinformatics*, 18(1), 529
[11] Ferreira, Tiago and Wayne Rasband, The Image J User Guide, http:// imagej.nih.gov/ij/docs/user-guide.pdf, date of access at 1 February 2019
[12] Priyadarsini, Kavirayani Indira. *Molecules*, **2014**, 19 (12), 20091-20112.

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