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GC-MS analysis of rhizome ethanol extracts from *Curcuma aeruginosa* accessions and their efficiency activities as anticancer agent

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Abstract. *Nurcholis W, Khumaida N, Bintang M, Syukur M. 2021. GC-MS analysis of rhizome ethanol extracts from Curcuma aeruginosa accessions and their efficiency activities as anticancer agent. Biodiversitas 22: 1179-1186.* This work aimed to evaluate the bioactive compounds and anticancer activity in rhizome extract of ten *Curcuma aeruginosa* accessions to explore their pharmacological values. The GC-MS analysis was used to identify bioactive compounds. The cytotoxic activity was performed against MCF-7 (Human breastadeno carcinoma) and Vero cell lines using MTT assay. The GC-MS analysis revealed 71 of the compounds as sesquiterpenes (36), monoterpenes (20), phenolics (5), diterpenes (4), phenanthrene (1), tetrapeptides (1), oxazole (1), triazine (1), piperidine (1), and oxygenated hydrocarbons (1). The isocurcumenol was the most dominant metabolite in ethanol extract of *C. aeruginosa* rhizome, with the highest produced by KP accession (22.01%) followed MD accession (21.12%). However, camphor and β-elemene were the metabolites produced by all accessions studied. In the Vero cell line as a normal cell, the cytotoxic activity varied from 13.28% (MD) to 45.17% (PW). Furthermore, the cytotoxic activity ranged from 1.16% (LC) to 49.70% (MD) against the MCF-7 cell line. The highest anticancer activity was produced in MD accessions; thus, it can be used as a source of quality raw materials for the pharmaceutical and food industry. Besides that, it can also be further developed to obtain superior varieties through plant breeding programs.

Keywords: Agricultural biochemistry, bioactivities, chemometric analysis, profiling metabolite, volatile compounds

INTRODUCTION

Curcuma aeruginosa, a perennial plant belonging to the Zingiberaceae family, is used as medicinal plant. It is a native of Myanmar and distributed to Indonesia, Malaysia, Indochina, and Ceylon (Sirirugsa et al. 2007). In traditional medicine, *C. aeruginosa* is used to treat many diseases such as flatulence, dysentery, dyspepsia, and gastritis (Theanphong et al. 2015). The *C. aeruginosa* plant contains many biologically active compounds obtained from rhizomes and leaves, used as a possible medicine for various human diseases (Moektiwardoyo et al. 2014; Srivilai et al. 2018; Suphrom et al. 2012). The phytochemical compounds contained in the *C. aeruginosa* contribute to its pharmacological properties. *C. aeruginosa* contains several bioactive compounds such as flavonoid, phenolic acids (Nurcholis et al. 2016b), curcumin, dimethoxy-curcumin and bisdemethoxycurcumin (Nurcholis et al. 2019, 2016a), germacrone (Hossain et al. 2015; Srivilai et al. 2018), isocurcumenol, zederone, curcumenol, dehydrocurdione, zedoarondiol (Suphrom et al. 2012), tropolone, eucalyptol, and curcumol (Fitria et al. 2019). The reported biological activities of *C. aeruginosa* and its metabolites include antimicrobial (Akarchariya et al. 2017), analgesic effect (Reanmongkol et al. 2006), anti-androgenic (Suphrom et al. 2012), uterine relaxant effect (Thaina et al. 2009), antioxidant (Nurcholis et al. 2017), antinociceptive (Hossain et al. 2015), hair growth promotor (Pumthong et al. 2012), and anticancer (Fitria et al. 2019). Therefore, it is crucial to select *C. aeruginosa* accessions with high phytochemical content and high pharmacological activities, which can be developed for plant breeding programs.

The composition and bioactive contents of medicinal plants can be affected by different factors, including genotypes (Batubara et al. 2020) and environmental factors (Mahajan et al. 2020; Ncube et al. 2012). The polyphenol and curcuminoids contents of *C. aeruginosa* accessions are varied by geographic location (Nurcholis et al. 2016b, 2016a), but whether this variation is due to environmental differences or genetic variability is unclear. Recently, the GC-MS has been well established to identify different metabolites from plant extracts (Ghimire et al. 2017; Ukwubile et al. 2019). GC-MS analysis of *C. aeruginosa* essential oils identified monoterpenes and sesquiterpenes compounds associated with antibacterial activity (Akarchariya et al. 2017). Several metabolites such as terpenoids, organic acids, sterols, sugars, and fatty acids have been reported from the different extracts (methyl tert-butyl ether, methanol/chloroform) of *C. aeruginosa* using GC-MS analysis (Simoh and Zainal 2015). However, the identification of metabolites from *C. aeruginosa* accessions in ethanol extract associated with an anticancer activity using GC-MS and chemometric analyses is considerably limited.

Due to the importance of *C. aeruginosa* extract, it is necessary to investigate metabolites composition across various accessions related to pharmacological activities. Therefore, the present research focused on metabolites identification from the ethanol extract of different *C. aeruginosa* accessions and further evaluated for anticancer activity against MCF-7 cell line. We used the same soil and environment for the growth of *C. aeruginosa* rhizome; thus, the different accession results are a direct reflection of the genetic diversity. Profiling metabolites and cytotoxicity data were used to classify *C. aeruginosa* accessions using chemometric analysis. This result also showed how to choose the elite accessions to develop commercially grown varieties of *C. aeruginosa*.

MATERIALS AND METHODS

Plant material and extraction

A total of 10 *C. aeruginosa* fresh rhizome accessions obtained from various regions of Indonesia were collected in February 2015 and further identified by expert from the Tropical Biopharmaca Research Center, IPB University, Indonesia (Table 1). The rhizome of sample collection was then cultivated at the Tropical Biopharmaca Research Center in Bogor City (106°42'53.22" E, 6°32'25.47" N), West Java Province, in Indonesia at an altitude of 142.60 m and arranged with three replications using a completely randomized design. Rhizome samples were planted in the spacing of 50 cm x 50 cm and grown under the same conditions in latosol soil with contained 0.15% N, 1.52% organic C, and pH of 4.5 - 5. The soil was prepared by 1 kg of cow manure per planting hole two weeks before planting. All rhizomes were harvested at nine months after planting in August 2016. In this work, all fresh rhizomes were cut and dried until the moisture content was ≤ 10% and when subjected to extraction, powdered at 80 mesh.

The rhizome powder was extracted using the maceration technique described in our previous research (Nurcholis et al. 2015). Briefly, the sample powder (25 g) were extracted at room temperature with 70% ethanol of 250 ml for 24 h. The solution sample was filtered with the Whatman filter paper of number 4. Then, the solution was evaporated at 50°C using a rotary evaporator (BUCHI, R-250, Switzerland). The extracted result was used for GC-MS and anticancer analysis.

GC-MS analysis

The ethanol extract of each accession was analyzed for the metabolite profile using gas chromatography-mass spectrometry (GC-MS). Previously, the sample extract (1 g) was extracted with 10 ml of hexane and then sonicated for 30 min. The filtered hexane solution (2 µl) was used for GC-MS analysis (Agilent GC 7890 series and Agilent MS 6950 series, USA) equipped with HP-5ms GC J&W capillary column (30 m x 0.25 mm i.d. and 0.25 µm film thickness). The helium was used as carrier gas with a 1 ml/min flow rate and injector temperature 250°C. The column oven temperature was programmed as follows: 40°C (hold for 2 min) to 50°C/min to 280°C as final temperature (hold for 2 min). The MS was operated at 70 eV, and the mass range scanned was 35 - 500 amu. All accessions were analyzed once without replication. The identification of compounds, including name, chemical structure, and molecular weight, was determined by adjusting the chromatogram spectra peaks with the known compounds in the NIST databases and PubChem data.

MTT assay

The anticancer analysis was performed using breast cancer cell line MCF-7 (ATCC HTB 22) and normal Vero cells (ATCC CCL 81) as a comparison. The cytotoxic activity of sample extract was measured colorimetrically using MTT (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) according to (Vijayarathna and Sasidharan 2012) protocols with slight modification. The MTT assay is often used to measure how cells are alive and to detect the toxicity of cells. This test is attributed to the loss of a yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. Briefly, the MCF-7 and Vero cells were cultured in Dulbecco's minimum Eagle's medium (Gibco, Rockville, MD, USA) and supplemented with fetal bovine serum (5%; Sigma-Aldrich, St. Louis, MO, USA), 100 µg/mL penicillin (Gibco, Rockville, MD, USA) and 100 µg/mL streptomycin (Gibco, Rockville, MD, USA). Cells were grown at a concentration of 5000 cells in 100 µL of growth medium. The extract (250 µg/ml) was added after the cells reached 50% confluent (24 hours). The MTT test was carried out on the third day by adding 10 µL of MTT per test well and incubating 4 hours at 37°C. Formazan crystals dissolved in ethanol. The absorbance value reading was carried out at a wavelength of 595 nm with a microplate reader (Bio-Rad 680, USA). Cytotoxicity value was calculated based on the percentage of inhibition of cell growth.

Table 1. The location origin and name of the ten *C. aeruginosa* accessions.

Origin/Location	Accession code	Geographical information		
		Altitude (m)	Latitude (N)	Longitude (E)
Klewer, Central Java	KL	96	7°35'05.66"	110°49'45.38"
Pakem, Yogyakarta	PK	424	7°39'55.46"	110°25'11.30"
Beringharjo, Yogyakarta	BH	115	7°47'56.40"	110°22'01.56"
Gunung Kidul, Yogyakarta	GK	180	7°58'04.87"	110°36'09.67"
Kulonprogo, Yogyakarta	KP	20	7°56'25.03"	110°14'20.30"
Purworejo, Central Java	PW	56	7°44'25.35"	110°01'59.00"
Madura, East Java	MD	4	7°02'48.90"	112°43'47.32"
Cirebon, West Java	LC	1	6°48'17.09"	108°48'06.04"
Bogor, West Java	CB	148	6°32'35.89"	106°41'22.41"
Muara Bungo, Jambi	MB	65	1°37'00.61"	102°22'16.28"

Data analysis

Statistical analysis of antiproliferative data was performed by analysis of variance (ANOVA) followed by the Scott-Knott test to identify significant differences between *C. aeruginosa* accessions with R using package 'ExpDes'. Significant differences between accessions were determined at $p \leq 0.05$. R was used with package 'pheatmap' and 'factoextra' for hierarchical cluster analysis (HCA)-heatmap dendrogram and principal component analysis (PCA), respectively. Graph of figure was generated using GraphPad Prism 8 for masOS (GraphPad Software Inc., San Diego, California, USA) Version 8.4.3.

RESULTS AND DISCUSSION

Metabolite compositions

Seventy-one metabolite compounds in the ethanolic extract of 10 *C. aeruginosa* accessions were successfully detected using GC-MS analysis. Those phytochemicals were identified based on retention time, peak area, and molecular formula (Table 2). From ten different extracted *C. aeruginosa* accessions the identified compounds were categorized into different groups (Figure 1) i.e sesquiterpenes (36), monoterpenes (20), phenolics (5), diterpenes (4), phenanthrene (1), tetrapeptides (1), oxazole (1), triazine (1), piperidine (1), and oxygenated hydrocarbons (1). Details of each compound were presented in Table 2.

In detail, the ethanolic extract of *C. aeruginosa* accessions is mainly composed of sesquiterpenes (19.86 - 43.72%) and monoterpenes (6.3 - 25.86%). These findings correspond with research by Akarchariya et al. (2017), who reported the sesquiterpenes (45.81%) and monoterpenes (45.55%) as major components in essential oils of *C. aeruginosa* rhizome from Thailand. But, the monoterpenes were considered low than the earlier reported. The results found presented four compounds as dominant monoterpene metabolites namely eucalyptol (3.12-9.20%), camphor (2.66-6.66%), 1-sarvone oxide (2.15-9.33%), and saussurea lactone (7.58-7.85%). Meanwhile, the dominant sesquiterpenes are β-elemene (1.16-2.67%), santonin (9.86-13.45%), herbertenolide (1.29-14.1%), epicurzerenone (2.05-12.38%), isocurcumenol (14.79-22.01%), germacrene B (1.82-3.68%), α-cadinene (0.50-5.76%), α-guaiene (0.08-2.54%), α-farnesene (1.26-2.96%), 1,5,9,9-tetramethyl-2-oxatricyclo[6.4.0.0(4,8)]dodecane (14.17%), 4,8-dimethyl-6-phenylazulene (1.19-2.26%), β-gurjunene (0.82-2.19%), and lactarolide A (2.05%). The most dominant metabolite in the ethanol extract of *C. aeruginosa* rhizome is isocurcumenol, with the highest production produced by KP accession. The isocurcumenol was detected in nine accessions except for CB accession.

However, the metabolites produced by all accessions studied are camphor and β-elements, which are monoterpenes and sesquiterpenes compounds, respectively. Several researchers have reported the results of profile metabolite content in *C. aeruginosa* rhizome in different extracts based on GC-MS analysis. The study from Akarchariya et al. (2017) reported that the major components of essential oils of *C. aeruginosa* rhizome from Thailand are camphor (29.39%) and germacrone (21.21%). The work from Kamazeri et al. (2012) showed that the essential oils of *C. aeruginosa* from Malaysia contain different major compounds identified as cycloisolongifolene, 8,9-dehydro formyl (35.29%) and dihydrocostunolide (22.51%). Meanwhile, different metabolites were identified by Simoh and Zainal (2015) in various extracts, such as methenolone (16.64%), cycloisolongifolene, 8,9-dehydro-9-formyl- (15.93%), labd-13-en-15-oic acid,8,12-epoxy-12-hydroxy-γ-lactone (10.77%), propiolic acid, 3-(1-hydroxy)-2 isopropyl-1,5-methylcyclohexyl) (7.84%), 4-oxo-β-isodamascol (5.17%), velleral (3.11%) and Z-α-farnesene (2.00%) in methyl tert-butyl ether extract, α-D glucopyranoside, 1,3,4,6 tetrakis-O-(TMS) (trimethylsilyl)-β-D-fructofuranosyl 2,3,4,6-tetrakis-O-(TMS)- (38.08%), d-glucose, 2,3,4,5,6-pentakis-O-(TMS)-, O-methyloxime (14.61%), D-fructose, 1,3,4,5,6-pentakis-O-(TMS)-, O-methyloxime (5.28%), isocitric acid (TMS) (3.06%), oxalic acid, bis (TMS) ester (2.96%), hexadecanoic acid, TMS ester (2.16%), citric acid, ethyl ester, tri-TMS (1.91%) and butanedioic acid, [(TMS) oxy]-, bis (TMS) ester (1.14%) in methanol-chloroform extract, and cycloisolongifolene, 8, 9-dehydro −9-formyl (15.70%), propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl) (11.09%), stearic acid, TMS ester (2.78%), hexadecanoic acid, TMS ester (2.33%), oleic acid, TMS ester (1.62%), curzerene (1.56%); Z-α-farnesene (1.52%), germacrone (1.41%) and β-elemene (1.33%) in chloroform extract. Therefore, it can be shown that, based on GC-MS examination, the form of extract and the source of the raw materials decide the quantity and quality of metabolites in the *C. aeruginosa* rhizome.

In this study, a total of 5 phenolics were also identified. Xanthotoxin, coumarin 138, trimethyl (2,6 ditert. -butylphenoxy) silane, 4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene, and trans-longipinocarveol were detected. Xanthotoxin was highest detected in accession GK and MB but lowest identified in accession PK. Accession PW and MB contained coumarin 138. Meanwhile, 4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene and trans-longipinocarveol compounds were detected in accessions GK and KL, respectively. Xanthotoxin shows antidepressant (Kowalczyk et al. 2021), anticancer (Mirzaei et al. 2017; Zhang et al. 2018), anti-inflammatory (Lee et al. 2017), and anticonvulsant (Zagaja et al. 2015, 2016) activities.

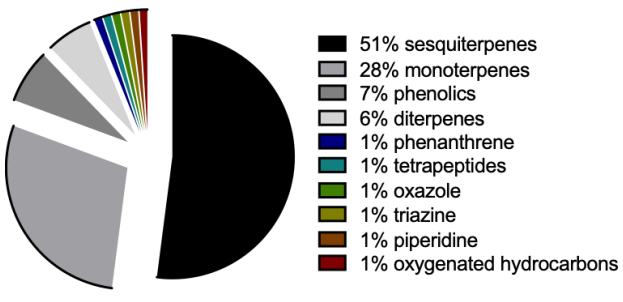

Figure 1. Pie Diagram displays the percentage of compound groups found in 10 *C. aeruginosa* accessions.

Table 2. Volatile compounds identified from ethanol extract of *C. aeruginosa* rhizome accessions.

Compounds	Group compounds	MF	MW	RT	%Total in accessions									
					KL	PK	BH	GK	KP	PW	MD	LC	MB	CB
Xanthotoxin	Phenolics	$C_{12}H_8O_4$	216	15.18		8.66		14.69					13.24	
Coumarin 138	Phenolics	$C_{14}H_{15}NO_2$	229	15.19						17.66			1.2	
1,4,5,8,9,12-Hexahydrotriphenylene	Phenanthrene	$C_{18}H_{12}O_6$	324	16.86	3.49									
Trimethyl (2,6 ditert.-butylphenoxy) silane	Phenolics	$C_{17}H_{30}OSi$	279	16.99	1.33									
6-Butyltetralin	Tetrapeptides	$C_{14}H_{20}$	188	17.88	5.54									
4,5-Dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene	Phenolics	$C_{14}H_{20}$	188	17.89				3.72						
Valerenol	Sesquiterpenes	$C_{15}H_{24}O$	220	18.70		2.4								
trans-Longipinocarveol	Phenolics	$C_{15}H_{24}O$	220	18.70	1.93									
4-Phenylthioacetophenone	Oxazole	$C_{14}H_{12}OS$	228	19.50	0.41									
Cymetrin	Triazine	$C_8H_{15}N_5S$	213	19.50		0.55								
2-Piperidone	Piperidine	C_5H_9NO	99	19.90		0.61		0.18						
2-Nonadecanone	Oxygenated hydrocarbons	$C_{19}H_{38}O$	282	20.32										0.36
(+)-Camphene	Monoterpenes	$C_{10}H_{16}$	136	6.29										0.09
β-Pinene	Monoterpenes	$C_{10}H_{16}$	136	6.69				0.22						0.08
Eucalyptol	Monoterpenes	$C_{10}H_{18}O$	154	7.47	3.71	3.56	3.87		4.69	5.43	9.2	3.49	3.79	3.12
Camphor	Monoterpenes	$C_{10}H_{16}O$	152	9.05	3.19	3.32	3.61	3.58	3.84	4.3	6.66	2.66	3.14	2.64
Camphene	Monoterpenes	$C_{10}H_{16}$	136	9.37	0.71			0.86					0.7	
Isononyl acrylate	Monoterpenes	$C_{13}H_{20}O_2$	208	9.37						1.02				
α-Pinene	Monoterpenes	$C_{10}H_{16}$	136	9.80		0.37								
Cyclopropane, 1-(1-methylethenyl)-2-(2-methyl-1-propenyl)-, (1R-trans)-	Monoterpenes	$C_{10}H_{16}$	136	9.80									0.32	
Terpineol	Monoterpenes	$C_{10}H_{18}O$	154	9.81				0.5						
Carvone	Monoterpenes	$C_{10}H_{14}O$	150	10.42	0.12									0.09
2-Carene	Monoterpenes	$C_{10}H_{16}$	136	12.09										0.09
4-Carene	Monoterpenes	$C_{10}H_{16}$	136	12.09		0.09								
Isoterpinolene	Monoterpenes	$C_{10}H_{16}$	136	12.10	0.11									
α-Terpinene	Monoterpenes	$C_{10}H_{16}$	136	12.12				0.26						
(+)-gamma-cadinene	Monoterpenes	$C_{15}H_{24}$	204	13.88										0.19
β-Ionene	Monoterpenes	$C_{13}H_{20}O$	192	16.32								1.69	0.09	
1-Carvone oxide	Monoterpenes	$C_{10}H_{14}O_2$	166	17.17	7.54	3.34	9.33	5.45	2.5	2.37	2.15			
Saussurea lactone	Monoterpenes	$C_{15}H_{22}O_2$	234	17.17		7.58					7.85			
1,1,3,4,4,6-Hexamethyltetralin	Monoterpenes	$C_{16}H_{24}$	216	18.45				0.88						
2-Isopropylidene-3-methylhexa-3,5-dienal	Monoterpenes	$C_{10}H_{14}O$	150	18.49								0.98		
δ-Elemene	Sesquiterpenes	$C_{15}H_{24}$	204	12.11									0.17	
β-Elemene	Sesquiterpenes	$C_{15}H_{24}$	204	12.80	1.51	1.46	1.75	1.86	1.72	2.67	2.36	1.2	2.14	1.16
Caryophyllene	Sesquiterpenes	$C_{15}H_{24}$	204	13.24										0.13
(E)-β-Farnesene	Sesquiterpenes	$C_{15}H_{24}$	204	13.50										0.29
+α-Amorphene	Sesquiterpenes	$C_{15}H_{24}$	204	13.87	0.22									
Epizonarene	Sesquiterpenes	$C_{15}H_{24}$	204	13.87				0.33						
Cyclo-γ-Cadinene	Sesquiterpenes	$C_{15}H_{24}$	204	13.87		0.2		0.27						
Aromadendrene	Sesquiterpenes	$C_{15}H_{24}$	204	14.08								1.57		
Cyclosativene	Sesquiterpenes	$C_{15}H_{24}$	204	14.08	1.33									
Allo-Aromadendrene	Sesquiterpenes	$C_{15}H_{24}$	204	14.08					2					
β-Selinene	Sesquiterpenes	$C_{15}H_{24}$	204	14.08			1.6	1.24		1.52	1.97			1.27
β-Guaiene	Sesquiterpenes	$C_{15}H_{24}$	204	14.08									1.21	
α-Selinene	Sesquiterpenes	$C_{15}H_{24}$	204	14.17				0.61					0.22	
δ-Cedrol	Sesquiterpenes	$C_{15}H_{26}O$	222	14.35										0.21
α-Amorphene	Sesquiterpenes	$C_{15}H_{24}$	204	14.35		0.21								
α-Copaene	Sesquiterpenes	$C_{15}H_{24}$	204	14.35									0.22	
α-Calacorene	Sesquiterpenes	$C_{15}H_{20}$	200	14.60	0.15	0.15								
β-Caryophyllene	Sesquiterpenes	$C_{15}H_{24}$	204	14.76									0.58	
Santonin	Sesquiterpenes	$C_{15}H_{18}O_3$	246	15.19								11.21	13.45	9.86
Herbertenolide	Sesquiterpenes	$C_{15}H_{18}O_2$	230	15.19					14.1				1.29	
Epicurzerenone	Sesquiterpenes	$C_{15}H_{18}O$	230	15.20	12.38	2.24		3.09	2.42	2.05	13.4		7.01	2.18
Isocurcumenol	Sesquiterpenes	$C_{15}H_{22}O_2$	234	15.63	14.79	15.44	21	17.08	22.01	19.72	21.12	17.49	5.06	
Germacrene B	Sesquiterpenes	$C_{15}H_{24}$	204	15.90	3.68								1.82	
α-Cadinene	Sesquiterpenes	$C_{15}H_{24}$	204	15.94								5.76	0.5	1.04

Caryophyllene	Sesquiterpenes	$C_{15}H_{24}$	204	16.09	1.06							0.84		
Ishwarane	Sesquiterpenes	$C_{15}H_{24}$	204	16.38			1.74					0.64		
α-Guaiene	Sesquiterpenes	$C_{15}H_{24}$	204	16.40						2.54		0.08		
α-Farnesene	Sesquiterpenes	$C_{15}H_{24}$	204	16.50	1.34	1.26					2.96			
1,5,9,9-Tetramethyl-2-oxatricyclo[6.4.0.0(4,8)]dodecane	Sesquiterpenes	$C_{15}H_{26}O$	222	16.76			14.17							
4,8-Dimethyl-6-phenylazulene	Sesquiterpenes	$C_{18}H_{16}$	232	17.38						1.19			2.26	
β-Ionene	Sesquiterpenes	$C_{13}H_{18}$	174	18.03									0.85	
β-Gurjunene	Sesquiterpenes	$C_{14}H_{20}$	188	18.24				0.82			2.19			
1,10: 4,5-Diepoxy-7(11)-germacren-8-one	Sesquiterpenes	$C_{15}H_{22}O_3$	250	18.70			1.41							
Lactarolide A	Sesquiterpenes	$C_{15}H_{22}O_5$	282	19.18			2.05							
Versalide	Sesquiterpenes	$C_{18}H_{26}O$	258	19.50									0.61	
Xeniaphyllenol B	Diterpenes	$C_{20}H_{32}O_2$	305	11.33				0.17						
(10-[(Acetyloxy) methyl]-9-antrhryl) methyl acetate	Diterpenes	$C_{20}H_{18}O_4$	322	15.90		3.64						1.52		
Columbin	Diterpenes	$C_{20}H_{22}O_6$	358	22.63				0.96						
Labd-7,13-(E)-dien-15-ol	Diterpenes	$C_{20}H_{34}O$	290	22.65			1.95							

Note: MF: molecule formula; MW: molecule weight (g/mol); and RT: retention time (min)

Regarding diterpenes compound, a total of 4 compounds were identified. Xeniaphyllenol B, (10-[(acetyloxy) methyl]-9-antrhryl) methyl acetate, columbin, and labd-7,13-(E)-dien-15-ol were detected. The distribution between accessions of these compounds showed that the accessions GK contained xeniaphyllenol B, accession PK and MB contained (10-[(acetyloxy) methyl]-9-antrhryl) methyl acetate, accession GK contained columbin, and accession BH contained labd-7,13-(E)-dien-15-ol. Columbin is known to possess anticancer (Kohno et al. 2002) and anti-inflammatory (Ibrahim Abdelwahab et al. 2012) effects.

The following six other group compounds namely phenanthrene (1,4,5,8,9,12-hexahydrotriphenylene), tetra-peptides (6-butyltetralin), oxazole (4-phenylthioaceto-phenone), triazine (cymetrin), piperidine (2-piperidone), and oxygenated hydrocarbons (2-nonadecanone) were also detected in accessions studied (Table 2). Accession KL contained 1,4,5,8,9,12-hexahydrotriphenylene, 6-butyltetralin, and 4-phenylthioacetophenone compounds. Cymetrin and 2- nonadecanone were recorded in accession PK and CB, respectively. 2-Piperidone was detected in two accessions (PK and GK).

Anticancer activity

Cytotoxic activity in ethanol extract of 10 *C. aeruginosa* accessions against MCF-7 and Vero cell lines is presented in Figure 2. In the Vero as a normal cell, the accession PW showed maximum cytotoxic activity with a value of 45.17%. This value not significantly different from doxorubicin (45.93%), accession KP (44.89%), GK (39.74%), and KL (37.64%). In comparison, accession MD presented the lowest cytotoxic activity with a value of 15.13%. Meanwhile, in the MCF-7, the cytotoxic activity ranged between 3.04% (accession CB) to 53.65% (accession MD). All accessions studied showed significantly different from doxorubicin (65.05%) at $p \leq 0.05$. A successful anticancer is that it can destroy cancer cells, but must have a limited impact on normal growth of cells (Safarzadeh et al. 2014). Results suggested selecting the accession MD to continue developing *C. aeruginosa* varieties with high anticancer activity through a breeding program. Atun et al. (2010) reported that methanol extract and hexane and chloroform fractions of *C. aeruginosa* have potent anticancer activity against the MCF-7 and Ca Ski cell lines. However, recently no study is found on ethanolic extract of *C. aeruginosa* for anticancer potential against the MCF-7 cell. Consequently, the results of this study have shown that the ethanolic extracts of *C. aeruginosa* can be produced as drug molecules for cancer disease.

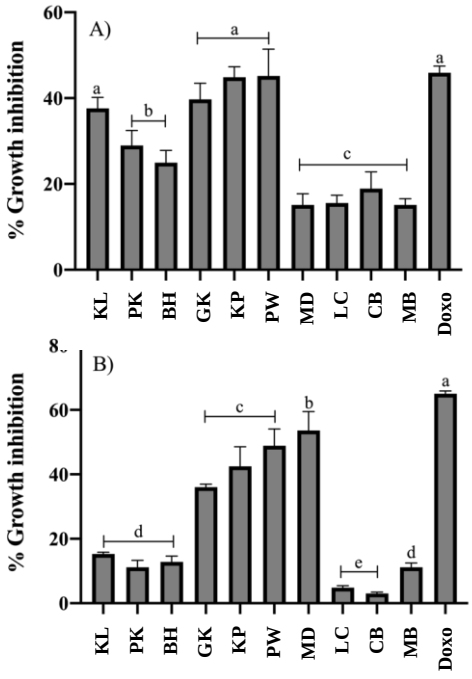

Figure 2. Antiproliferative activity of the ethanol extract from the rhizome of *Curcuma aeruginosa* accessions against (A) Vero and (B) MCF-7 cell lines. Doxo, doxorubicin as a positive control. Values show the mean ± SD for n = 3. According to the Scott-Knott test at $p \leq 0.05$, two independent studies (A and B) with different superscripts (a, b, c, d, e) differ significantly.

Multivariate analysis

In this work, the principal component analysis (PCA) and hierarchical cluster analysis (HCA)-heatmap dendrogram in *C. aeruginosa* studied accessions were used for multivariate analysis, namely chemometric. The chemometric allows for the quantification and enhancement of the understanding of metabolites information and the association of quality traits to analytical instruments data (Batubara et al. 2020). It has been used to determine the chemical components in plants that contribute to its medicinal benefits (Abbasi et al. 2018; Guo et al. 2020; Windarsih et al. 2019).

PCA analysis revealed that the first two components (shown in Figure 3) accounted for a total of 35.2% of the data variance explained. The individual score plot (Figure 3A) and loading plot (Figure 3B) represent the significant differences between the accessions studied. The diversity of data explained by PCA is low, so HCA analysis is often required to better describe the data (Khumaida et al. 2019; Péroumal et al. 2017). In chemometric analysis (PCA and HCA), it was resulted five clusters. Cluster 1 consisted of one accession viz. CB due to their high metabolite content of 4,8-dimethyl-6-phenylazulene (M62), (E)-β-farnesene (M36), δ-cedrol (M46), versalide (M67), (+)-gamma-cadinene (M27), 2-carene (M23), 2-nonadecanone (M12), (+)-camphene (M13), caryophyllene (M35), β-ionene (M63), and santonin (M51). Cluster 2 was composed one accession: KL. This accession presented the highest carvone (M22), 1,4,5,8,9,12-hexahydrotriphenylene (M3), isoterpinolene (M25), cyclosativene (M41), trans-longipinocarveol (M8), trimethyl (2,6 ditert.-butylphenoxy) silane (M4), 6-butyltetralin (M5), 4-phenylthioacetophenone (M9), +α-amorphene (M37), epicurzerenone (M53), camphene (M17), germacrene B (M55), and caryophyllene (M57) metabolites. Accession GK grouped in cluster 3. This group was characterized by high β-pinene (M14), α-selinene (M45), 4,5-dimethyl-1,2,3,6,7,8,8a,8b-octahydrobiphenylene (M6), epizonarene (M38), terpineol (M21), Columbin (M70), xeniaphyllenol B (M68), α-terpinene (M26), 1,1,3,4,4,6-hexamethyltetralin (M31), xanthotoxin (M1), and cyclo-γ-cadinene (M39). Cluster 4 consisted accession PK with characterized by high α-calacorene (M49), (10-[(acetyloxy) methyl]-9-antrhryl) methyl acetate (M69), 2-piperidone (M11), cymetrin (M10), 4-carene (M24), α-amorphene (M47), valerenol (M7), and α-pinene (M19) metabolites.

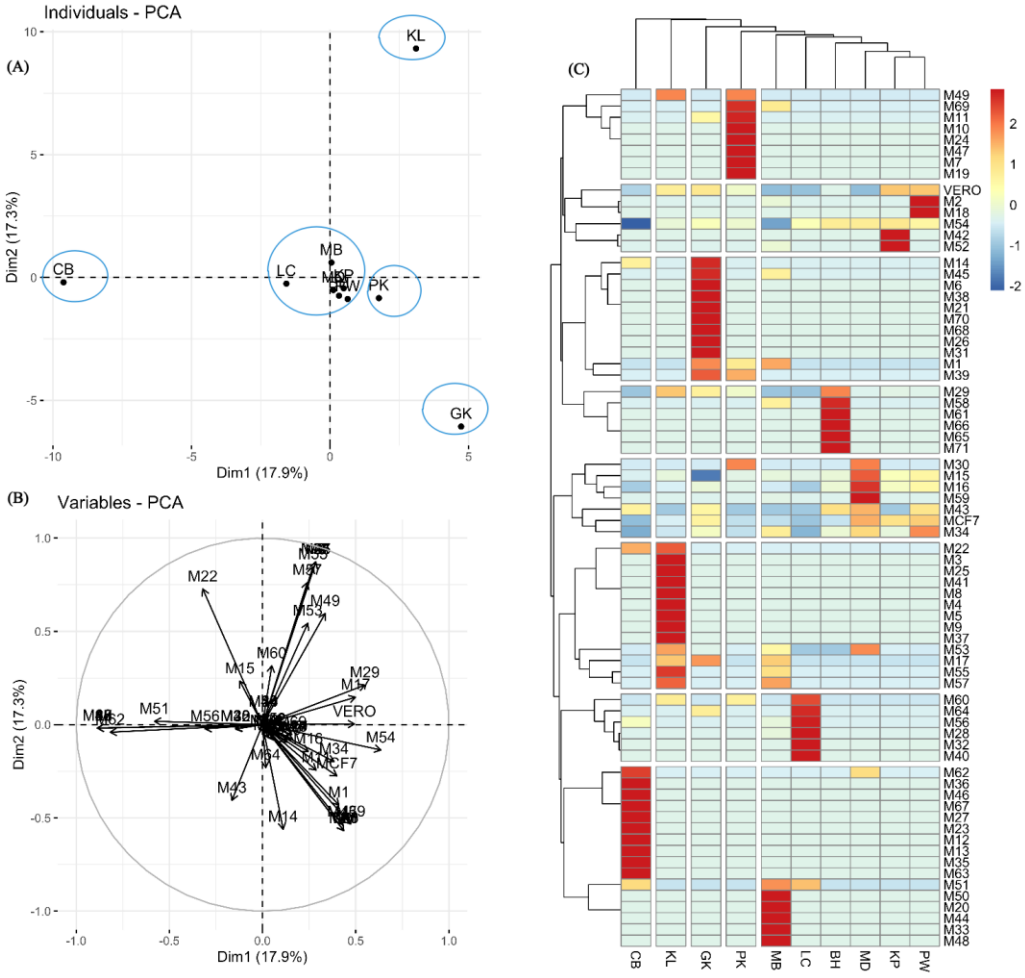

Figure 3. Score plot (A), loading plot (B) and HCA-heatmap dendrogram (C) of PCA for *C. aeruginosa* accessions using the metabolites (M1 - M71, see in Table 2) and cytotoxicity activities against Vero and MCF-7 cell lines matrix as input variables. The darker red, yellow and darker blue presented higher, moderate and lower metabolites contents and cytotoxic activities, respectively.

Cluster 5 represented MB, LC, BH, MD, KP, and PW accessions. Interestingly, accessions studied in cluster 5 were associated with metabolites and cytotoxic activities. The highest cytotoxic activities against MCF-7 cell line found in accession MD (53.65%) followed with accession PW (48.91%) and KP (42.52%), but the accessions PW (45.17%) and KP (44.89%) also high cytotoxic activity against Vero cell line. These accessions had high metabolites saussurea lactone (M30), eucalyptol (M15), camphor (M16), epicurzerenone (M53), and β-elemene (M34) and cytotoxic activity against the MCF-7 cell line, indicating the association of maximum metabolites with anticancer activity. Past research has shown these compounds from several medicinal plants to be useful as anticancer. Saussurea lactone was isolated from the roots of *Saussurea lappa*, which exhibited potent anticancer properties (Robinson et al. 2008). Yang et al. (2010) showed that the eucalyptol compound from the essential oil of *Amomum tsao-ko* had anticancer activity against HepG2 carcinoma cell line. The camphor compound found in the essential oil of *Origanum vulgare* has been shown to have anti-cancer activity (Elansary et al. 2018). Rahman et al. (2013) isolated epicurzerenone (curzerenone as synonym name) from *Curcuma zedoaria*, which exhibited cytotoxicity on Ca Ski, MCF-7, and HCT-116 cancer cell lines. Meanwhile, β-elemene has been reported to have anticancer activity for several types of cancer (Deng et al. 2020; Zhai et al. 2019). The future will combine the main target of new varieties development for *C. aeruginosa* as anticancer sources high metabolite content and anticancer activity. The results indicated that phytochemical parameters including saussurea lactone, eucalyptol, camphor, epicurzerenone, and β-elemene could be used as a selection parameter the development *C. aeruginosa* varieties in addition to anticancer activity through in the breeding program.

The results show that the ethanolic extract of *C. aeruginosa* accessions contains a variable pattern of sesquiterpenes, monoterpenes, phenolics, diterpenes, and others. Isocurcumenol was found as major compound of accessions studied. Camphor and β-elemene compounds were detected in majority of accessions studied. The chemometric analysis concluded that the saussurea lactone, eucalyptol, camphor, epicurzerenone, and β-elemene metabolites are responsible for anticancer activity against the MCF-7 cell line in ethanolic extract of *C. aeruginosa* accessions. Accession MD had the maximum anticancer activity; therefore, it can select this accession to continue developing *C. aeruginosa* varieties through the breeding program as a source of drug metabolites producing to treat cancer diseases.

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