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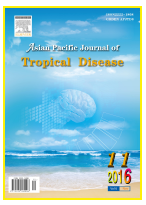

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Variability of curcuminoid content and lack of correlation with cytotoxicity in ethanolic extracts from 20 accessions of *Curcuma aeruginosa* RoxB

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ABSTRACT

Objective: To evaluate the contents of curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) and the cytotoxic activity in ethanolic extracts of 20 *Curcuma aeruginosa* RoxB. (*C. aeruginosa*) accessions collected from regions of Indonesia.

Methods: Curcuminoid compounds were investigated by high performance liquid chromatography with standards, and the cytotoxic activity was evaluated by the brine shrimp lethality test.

Results: Curcuminoids, curcumin, demethoxycurcumin and bisdemethoxycurcumin contents of *C. aeruginosa* accessions varied from 0.01% to 1.95% with the coefficient of variation (CV) of 27.36%, 0.01% to 1.45% with a CV of 30.56%, 0.01% to 0.47% with a CV of 25.16%, and 0.01% to 0.03% with a CV of 30.90%, respectively, which was higher in the Wonogiri accessions. All accessions of *C. aeruginosa* were found to be effective in general toxicity against brine shrimps.

Conclusions: The Wonogiri accession of *C. aeruginosa* could be selected as a high quality clone for curcuminoid production for an industry breeding program.

1. Introduction

Curcuma aeruginosa RoxB. (*C. aeruginosa*), namely temu hitam or temu ireng in Indonesia, is a medicinal plant that belongs to family Zingiberaceae[1]. Rhizomes of *C. aeruginosa* are used in traditional medicine to relieve stomach pain, enteritis, asthma, rheumatic problems, increase appetite, prevent obesity and serve as an anthelmintic. Many biological activities have been reported for *C. aeruginosa,* such as an anti-inflammatory agent[2], a drug to increase numbers of thrombocytes in dengue fever treatment[3], an anti-androgenic[4], antimicrobial agent[5], a platelet-activating factor and antagonists for treatments of immunological and inflammatory disorders[6], an antinociceptive[7], an antioxidant[8,9] and as an anticancer agent[10]. Curcuminoids are natural polyphenolic compounds and bioactive compounds found in the rhizome of *C. aeruginosa*[11]. The chemical compositions of curcuminoids are curcumin, demethoxycurcumin and bisdemethoxycurcumin, which play important roles in several pharmacological activities. Curcuminoids have been found to possess diverse pharmacological activities, such as antioxidant[12,13], anti-inflammatory[14,15], neuroprotection[16], antidiabetic[17] and anticancer[18,19]. Among activities of curcuminoids, cytotoxic activity is typically interesting, because it can be used for anticancer drug opportunity in industry. Based on their pharmacological properties, curcuminoids are important compounds in the pharmaceutical industry.

Pharmacological activities of medicinal plants are influenced by the quality of the raw material used[20]. In Indonesia, we have not yet found a quality standard for *C. aeruginosa*[21]. In order to be used by the pharmaceutical industry, uniform genotypes need to be bred. There is a need to investigate *C. aeruginosa* from various regions of Indonesia, which may help to find accessions with the best curcuminoid content and cytotoxic activity to be used in an initial breeding program. Therefore, the objective of the present study was to evaluate the curcuminoid (curcumin, demethoxycurcumin and bisdemethoxycurcumin) contents and the cytotoxic activity in ethanolic extracts of 20 *C. aeruginosa* accessions collected from different regions of Indonesia.

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2. Materials and methods

2.1. Plant materials

The fresh rhizomes of 20 accessions of *C. aeruginosa* were collected from different regions of Indonesia in February 2015 including East Java (5 accessions), Central Java (8 accessions), Yogyakarta (4 accessions), West Java (2 accessions) and Jambi (1 accession) (Table 1). Identification of the specimen accessions was confirmed by the Tropical Biopharmaca Research Center, Bogor Agricultural University. Voucher specimens have been deposited in the Tropical Biopharmaca Research Center, Bogor Agricultural University.

Table 1
Geographical collection sites of 20 accessions of *C. aeruginosa* in Indonesia.

Accession code	Region	Province	Latitude (S)	Longitude (E)	Altitude (m)
MD	Madura	East Java	7°02'48.90"	112°43'47.32"	4
KD	Kediri	East Java	7°50'39.52"	111°53'54.93"	489
PR	Ponorogo	East Java	7°51'51.47"	111°28'11.78"	106
PT	Pacitan	East Java	8°11'59.56"	111°06'13.34"	7
NW	Ngawi	East Java	7°29'52.21"	111°09'22.78"	345
KA	Karanganyar	Central Java	7°39'49.37"	111°08'01.93"	1113
SG	Sragen	Central Java	7°24'22.14"	111°07'12.84"	90
GD	Gede-Solo	Central Java	7°34'08.83"	110°49'54.53"	95
KL	Klewer-Solo	Central Java	7°35'05.66"	110°49'45.38"	96
SH	Sukoharjo	Central Java	7°44'41.62"	110°52'41.14"	111
WG	Wonogiri	Central Java	7°57'22.83"	110°59'37.51"	378
PW	Purworejo	Central Java	7°44'25.35"	110°01'59.00"	56
KN	Kendal	Central Java	7°00'55.14"	110°16'05.98"	78
PK	Pakem	Yogyakarta	7°39'55.46"	110°25'11.30"	424
BH	Beringharjo	Yogyakarta	7°47'56.40"	110°22'01.56"	115
KP	Kulonprogo	Yogyakarta	7°56'25.03"	110°14'20.30"	20
GK	Gunung Kidul	Yogyakarta	7°58'04.87"	110°36'09.67"	180
LC	Losari-Cirebon	West Java	6°48'17.09"	108°48'06.04"	1
CB	Ciampea-Bogor	West Java	6°32'35.89"	106°41'22.41"	148
MB	Muara Bungo	Jambi	1°37'00.61"	102°22'16.28"	65

2.2. Preparation of rhizomes and extraction

Rhizomes of *C. aeruginosa* were cut and dried for 5 days in the sun (moisture content, < 10%) and then powdered to the size of 100 mesh. The extraction procedure was performed by maceration according to our earlier research[22]. Briefly, 100 g of the powder was extracted with 1 000 mL of 70% (v/v) ethanol at room temperature for a period of 24 h and then filtered using Whatman paper filter No. 4. The whole process was repeated once (1 × 24 h). Finally, the crude extract was concentrated by evaporation (BUCHI, R-250, Switzerland) at 50 °C. These extracts (yield range, 7.92%–19.71% of dry weight) were then used in later experiments.

2.3. Analysis of curcuminoid content

The curcuminoid content in ethanolic extracts of *C. aeruginosa* accessions were measured by high performance liquid chromatography (HPLC)[23] using curcumin (1), demethoxycurcumin (2), and bisdemethoxycurcumin standards which were purchased from ChromaDex Inc. (Santa Ana, CA, USA). All solvents used were HPLC grade and obtained from Merck (Darmstadt, Germany). Briefly, 50 mg ethanolic extracts were sonicated with methanol (10 mL) for 1 h at room temperature. All extracts were pooled and injected into the HPLC (Shimadzu LC-20A series, Japan) equipped with a diode array UV detector and shim-pack VP-ODS C18 column (150 mm × 4.6 mm identification, 4.6-micrometer particle size) (Shimadzu, Tokyo, Japan). Elution was carried out at a flow rate 1 mL/min with acetonitrile as solvent A and 0.5% v/v acetic acid in water as solvent B, using a gradient elution in 0–30 min with 40%–75% of solvent A, 30–40 min with 100% of solvent B and monitored at 425 nm for quantitation of curcuminoids. Results were obtained by comparing with the standards. Curcuminoid (curcumin, demethoxycurcumin and bisdemethoxycurcumin) contents in ethanolic extracts of *C. aeruginosa* accessions were expressed as percentage (w/w) extract to weight basis.

2.4. Analysis of the cytotoxic activity

The cytotoxic activity in ethanolic extracts of *C. aeruginosa* accessions was evaluated by the brine shrimp lethality (BSLT) test according to the protocol of Meyer *et al.*[24]. Briefly, the extracts of samples were prepared by dissolving them in dimethyl sulfoxide (Merck, Germany) and adding to the seawater to make final concentrations of 50, 100, 500 and 1 000 μg/mL. A total of 10 brine shrimps were transferred into each vial containing seawater and extracts of samples at different concentrations. A control was run containing only dimethyl sulfoxide. After 24 hours of exposure, the percentage mortality was recorded and the results were subjected to Minitab 16 for LC_{50} calculation.

2.5. Statistical analysis

Results were expressed as mean ± SD (n = 3). The statistical analyses consisted of ANOVA with completely randomized design, relying on the Statistical Tool for Agricultural Research software, version 2.0.1. Tukeys's honest significant difference test was employed to detect the significance of differences (P < 0.05) between mean values. Pearson correlation coefficients were calculated among curcuminoid contents and the cytotoxic activity. Hierarchical cluster analysis was done based on Euclidean distances from curcuminoid contents and the cytotoxic activity data matrix.

3. Results

Variation was observed in curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) of 20 *C. aeruginosa* accessions (Table 2). Among recorded compounds, curcumin, demethoxycurcumin, bisdemethoxycurcumin and total curcuminoid were the most variable characteristics of the accessions of *C. aeruginosa* (the coefficient of variation, CV = 30.56%, 25.16%, 30.90% and 27.36% respectively). The content of curcuminoids varied between 0.01% in accessions MB and PR to 1.95% in accession WG. The accessions of CB, KD, KL, KN, KP, MD, and NW showed no detectable (0.01%) curcuminoid content. Curcumin levels ranged from 0.01% (accessions of GD, GK, KA, LC, PR, and SG) to 1.45% (accession WG), with the exception of accessions CB, KD, KL, KN, KP, MB, MD and NW, in which none was detected (< 0.01%). Demethoxycurcumin content ranged from 0.01 to 0.47%, with the accessions BH, GD, GK, KA, LC, MB, PK, PT, PW, and with SG having the lowest demethoxycurcumin content and the accession WG the highest, while accessions CB, KD, KL, KN, KP, MD, NW and PR showed no detectable content (< 0.01%). Higher

levels of bisdemethoxycurcumin were identified in WG (0.03%), closely followed by accessions SH (0.02%) and PW (0.01%). The levels of bisdemethoxycurcumin from accessions BH, CB, GD, GK, KA, KD, KL, KN, KP, LC, MB, MD, NW, PK, PR, PT and SG were too low to be detectable (< 0.01%). For total curcuminoid (curcumin, demethoxycurcumin, and bisdemethoxycurcumin) contents, the accession from WG showed a remarkable difference with the others ($P < 0.05$).

Table 2

Variation of curcuminoids compound (curcumin, demethoxycurcumin, bisdemethoxycurcumin) among different accessions of *C. aeruginosa*.

Accession code	Percentage content (w/w)*			
	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin	Curcuminoids
BH	0.02 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.03 ± 0.001^{c}
CB	nd	nd	nd	nd
GD	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.02 ± 0.001^{c}
GK	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.02 ± 0.001^{c}
KA	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.02 ± 0.001^{c}
KD	nd	nd	nd	nd
KL	nd	nd	nd	nd
KN	nd	nd	nd	nd
KP	nd	nd	nd	nd
LC	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.02 ± 0.001^{c}
MB	nd	0.01 ± 0.001^{c}	nd	0.01 ± 0.001^{c}
MD	nd	nd	nd	nd
NW	nd	nd	nd	nd
PK	0.02 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.03 ± 0.001^{c}
PR	0.01 ± 0.001^{c}	nd	nd	0.01 ± 0.001^{c}
PT	0.03 ± 0.024^{c}	0.01 ± 0.011^{c}	nd	0.04 ± 0.037^{c}
PW	0.03 ± 0.001^{c}	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	0.05 ± 0.001^{c}
SG	0.01 ± 0.001^{c}	0.01 ± 0.001^{c}	nd	0.02 ± 0.001^{c}
SH	0.57 ± 0.020^{b}	0.23 ± 0.025^{b}	0.02 ± 0.002^{b}	0.82 ± 0.048^{b}
WG	1.45 ± 0.145^{a}	0.47 ± 0.035^{a}	0.03 ± 0.004^{a}	1.95 ± 0.177^{a}
CV (%)	30.56	25.16	30.90	27.36

*: Values are shown as means ± SD of three replicate experiments; Different letters within columns indicate significant difference (Tukey, $P < 0.05$); nd: Not detectable (percentage content < 0.01%).

The cytotoxicity of the ethanolic extracts in different accessions of *C. aeruginosa* was evaluated using the BSLT for potency in preliminary screening for cytotoxins. The results of the BSLT were shown in Table 3. The percentage mortality of brine shrimp was found to be directly proportional to the concentration of the ethanolic extract used in all accessions of *C. aeruginosa* (Table 3). All accessions of *C. aeruginosa* were found to be toxic (LC_{50} < 1 000 μg/mL) at levels ranging from 57.32 μg/mL in the accession from NW to 670.96 μg/mL in the accession from KL. Among the recorded traits, percentage mortality in concentrations of 50, 100, 500 and 1 000 μg/mL and their LC_{50} values were the most variable characteristics of *C. aeruginosa* accessions (CV = 40.23%, 39.24%, 16.87%, 7.69% and 27.32%). The LC_{50} values of ethanolic extracts for accessions PW, NW, GD, and GK showed remarkable significant differences by the Tukeys's test ($P < 0.05$) (Statistical Tool for Agricultural Research, Version 2.0.1) as compared with the other accessions of *C. aeruginosa*.

Hierarchical cluster analysis based on Euclidean distances from curcuminoid content and cytotoxic activity data matrix grouped our 20 accessions of *C. aeruginosa* into three main categories, I, II and III (Figure 1). The accessions were grouped in terms of their dissimilarity. The first group (I) comprised accessions KL and PR, and represented accessions of *C. aeruginosa* with the lowest levels of curcuminoids and the cytotoxic activity. The second group (II) comprised accessions GD, NW, PW, GK, KA, KN, MB, MD, PT, SG and SH was characterized by undetectable curcuminoid levels (< 0.01%–0.82%), but had the highest cytotoxicity. The third group (III) comprised of BH, KP, CB, WG, KD, PK and LC accessions also had no detectable curcuminoids (< 0.01%–1.95%), but did have moderate cytotoxic activity.

Table 3

Variation of the cytotoxic activity on brine shrimp nauplii of ethanolic extracts among different accessions of *C. aeruginosa*.

Accession code	Mortality at different concentrations (μg/mL) (%)				LC_{50} (μg/mL)
	50	100	500	1 000	
BH	10.00 ± 10.00^{cde}	23.33 ± 5.77^{bc}	40.00 ± 10.00^{de}	83.33 ± 5.77^{abc}	392.13 ± 25.47^{bc}
CB	6.67 ± 11.55^{de}	16.67 ± 5.77^{bc}	40.00 ± 10.00^{de}	83.33 ± 15.28^{abc}	439.71 ± 31.65^{b}
GD	36.67 ± 5.77^{abc}	83.33 ± 11.55^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	60.90 ± 8.37^{e}
GK	33.33 ± 5.77^{abcd}	50.00 ± 10.00^{abc}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	83.20 ± 13.00^{e}
KA	23.33 ± 5.77^{bcde}	50.00 ± 10.00^{abc}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	90.92 ± 5.71^{de}
KD	6.67 ± 5.77^{de}	20.00 ± 10.00^{bc}	63.33 ± 15.28^{abcd}	96.67 ± 5.77^{ab}	260.16 ± 42.59^{bcde}
KL	5.00 ± 7.07^{e}	35.00 ± 21.21^{bc}	30.00 ± 0.00^{de}	65.00 ± 7.07^{cd}	670.96 ± 50.32^{a}
KN	3.33 ± 5.77^{e}	33.33 ± 5.77^{bc}	83.33 ± 5.77^{abc}	93.33 ± 11.55^{ab}	198.62 ± 16.39^{cde}
KP	5.00 ± 7.07^{e}	15.00 ± 7.07^{c}	45.00 ± 7.07^{cde}	85.00 ± 7.07^{abc}	408.81 ± 52.84^{b}
LC	6.67 ± 5.77^{de}	30.00 ± 10.00^{bc}	40.00 ± 17.32^{de}	100.00 ± 0.00^{a}	294.24 ± 29.42^{bcd}
MB	23.33 ± 5.77^{bcde}	33.33 ± 5.77^{bc}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	108.36 ± 11.74^{de}
MD	40.00 ± 17.32^{ab}	23.33 ± 32.15^{bc}	83.33 ± 11.55^{abc}	100.00 ± 0.00^{a}	120.87 ± 63.74^{de}
NW	36.67 ± 5.77^{abc}	86.67 ± 11.55^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	58.48 ± 6.90^{e}
PK	6.67 ± 5.77^{de}	26.67 ± 5.77^{bc}	63.33 ± 15.28^{abcd}	96.67 ± 5.77^{ab}	244.46 ± 16.92^{bcde}
PR	6.67 ± 5.77^{de}	10.00 ± 0.00^{c}	20.00 ± 10.00^{e}	76.67 ± 5.77^{bcd}	693.43 ± 136.38^{a}
PT	40.00 ± 10.00^{ab}	16.67 ± 15.28^{bc}	96.67 ± 5.77^{a}	100.00 ± 0.00^{a}	117.91 ± 41.79^{de}
PW	53.33 ± 5.77^{a}	60.00 ± 30.00^{ab}	86.67 ± 5.77^{ab}	100.00 ± 0.00^{a}	57.32 ± 23.12^{e}
SG	20.00 ± 10.00^{bcde}	26.67 ± 5.77^{bc}	90.00 ± 10.00^{ab}	100.00 ± 0.00^{a}	137.79 ± 16.65^{de}
SH	26.67 ± 11.55^{abcde}	23.33 ± 5.77^{bc}	80.00 ± 10.00^{abc}	100.00 ± 0.00^{a}	150.99 ± 3.22^{de}
WG	10.00 ± 0.00^{cde}	35.00 ± 12.21^{bc}	55.00 ± 19.50^{bcde}	60.00 ± 12.28^{d}	438.50 ± 26.43^{b}
CV (%)	40.23	39.24	16.87	7.69	27.32

Each value is expressed as mean ± SD of three replicate experiments. Different letters within columns indicate significant difference (Tukey, $P < 0.05$).

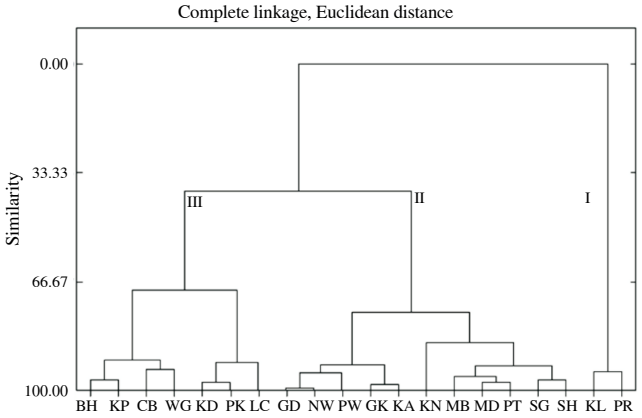

Figure 1. Dendrogram showing the similarity among different accessions of *C. aeruginosa* based on Euclidean distances from curcuminoids content and the cytotoxic activity data matrix.

4. Discussion

Our results showed variation in curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) content of *C. aeruginosa* accessions between 0.01% and 1.95% (Table 2). These curcuminoid concentrations were high when compared to that found by Bos *et al.*[11], with a percentage range of 0.02%–0.03%. Curcumin (0.01%–1.45%) and demethoxycurcumin (0.01%–1.45%) were found to be the dominant curcuminoids in all accessions of *C. aeruginosa*. This was similar with the results of Bos *et al.*[11] who concluded a curcumin and demethoxycurcumin content of 0.01% and 0.01%–0.02%, respectively, and found no detectable bisdemethoxycurcumin. Similarly, Jitoe *et al.*[25] reported values less than 25 μg per 4 mg for curcumin and demethoxycurcumin in CH_2Cl_2 extracts with no detectable bisdemethoxycurcumin. In our results, bisdemethoxycurcumin was detected in accessions from PW (0.01%), SH (0.02%) and WG (0.03%).

The results for the cytotoxicity of all accessions were positive, with toxicity levels (LC_{50} values) at concentrations lower than 1 000 μg/mL. Therefore, these results showed that all accessions of *C. aeruginosa* were potential candidates in anticancer therapy[24]. The main pharmacological effect of curcuminoid compounds is to serve as an anticancer agent. Other researchers have previously reported several anticancer activities in curcuminoid compounds. These included anticancer activities such as the inhibition growth of human breast cancer cells[26,27], prostate cancer cells[28,29], human lung cancer cells[30], human colon cancer cells[31] and liver cancer cells[32,33]. However, our results showed a low positive correlation between the cytotoxicity with curcumin, demethoxycurcumin, bisdemethoxycurcumin and curcuminoids, with Pearson correlation values of 0.161, 0.142, 0.053 and 0.155, respectively. According to these results, the curcuminoid compounds in ethanolic extracts of *C. aeruginosa* were not dominantly responsible for the observed cytotoxicity. The sesquiterpenoids compounds identified in the rhizome of *C. aeruginosa* have previously been reported by other researchers[34,35] who have identified several cases of cytotoxicity. Zhong *et al.*[36] reported that the sesquiterpenoid germacrone exhibited anticancer properties in human breast cancer. Similarly, other reports showed that sesquiterpenes were found to possess cytotoxicity in human cancer cell lines[37,38]. Therefore, further evaluations are required to identify the compound(s) that could be used as a potential anticancer therapy from *C. aeruginosa*.

In the present study, variation was observed in the curcuminoid content and the cytotoxicity of 20 *C. aeruginosa* accessions. The variations in curcuminoid content and the unrelated cytotoxicity among the evaluated *C. aeruginosa* accessions could be due to either genotype or growth environment or both. The highest concentrations of curcumin, demethoxycurcumin and bisdemethoxycurcumin were quantified at 1.45%, 0.47% and 0.03% (total curcuminoids 1.95%), respectively, as observed in the WG accession. In a breeding program for the pharmaceutical industry, the results provide important of information for selecting high quality *C. aeruginosa* plants for the large-scale production of curcuminoid compounds. However, our results show curcuminoids in ethanolic extracts of *C. aeruginosa* are not important as a future anticancer agent.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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