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Original Article

Xanthorrhizol contents, α-glucosidase inhibition, and cytotoxic activities in ethyl acetate fraction of *Curcuma zanthorrhiza* accessions from Indonesia

Waras Nurcholis[a,b,*], Ahmad Ajruddin Munshif[a], Laksmi Ambarsari[a]

[a] *Department of Biochemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Jawa Barat, Indonesia*
[b] *Tropical Biopharmaca Research Center, Bogor Agricultural University, Jawa Barat, Indonesia*

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ABSTRACT

Curcuma zanthorrhiza Roxb., Zingiberaceae, a species from Indonesia with xanthorrhizol as the major metabolite, has been used as a folk medicine in several of pharmacological activities. This work aimed to evaluate the xanthorrhizol contents, α-glucosidase inhibition, and cytotoxic activities in ethyl acetate fraction from accessions of *C. zanthorrhiza*. High-performance liquid chromatography investigated xanthorrhizol content with the standard. Pharmacological activities were evaluated by inhibition of α-glucosidase, the brine shrimp lethality test, and anticancer activity. The ethyl acetate fraction yield varied from 8.24% (Karanganyar) to 13.13% (Sukabumi). The xanthorrhizol contents were found to be in the range 43.55% to 47.99% with Ngawi and Wonogiri promising accessions having the lowest and highest value, respectively. IC_{50} value for α-glucosidase inhibition ranged from 339.05 μg/ml (Karanganyar) to 455.01 μg/ml (Ngawi). LC_{50} value for cytotoxic activities ranged from 33.25 μg/ml (Ngawi) to 42.28 μg/ml (Karanganyar) in brine shrimp lethality test, 3.10 μg/ml (Karanganyar) to 9.85 μg/ml (cursina-III) in Vero cell, and 1.17 μg/ml (Ngawi) to 6.83 μg/ml (Sukabumi) in MCF-7 cell. In this study, *C. zanthorrhiza* accessions have a high in xanthorrhizol contents and cytotoxic activities that showed a high potential of studied accessions for breeding programs on a commercial scale.

Introduction

Curcuma zanthorrhiza Roxb., also known as Java turmeric (namely "Temulawak" in Indonesia), is a well-known rhizomatous herb that belongs to the Zingiberaceae family (Kim et al., 2014; Nurcholis et al., 2016a). The origin of *C. zanthorrhiza* is Indonesia that distributed in Southeast Asian Region (Suksamrarn et al., 1994; Salea et al., 2014). Moreover, it is grown wild and cultivated widely in Malaysia, Thailand, Sri Lanka and Philippines (Devaraj et al., 2010). It has been traditionally used to overcome various diseases such as stomach diseases, liver disorders, constipation, bloody diarrhea, dysentery, children's fevers, hemorrhoids, and skin eruptions (Hwang et al., 2000). Xanthorrhizol (1), a bisabolane-type sesquiterpenoid compound with IUPAC name of 2-methyl-5-[(2*R*)-6-methylhept-5-en-2-yl]phenol, is the major bioactive constituent contained in rhizomes of *C. zanthorrhiza* (Oon et al., 2015). In the literature, xanthorrhizol of *C. zanthorrhiza* rhizomes are considered to possess anticancer (Kang et al., 2009; Kim et al., 2013), antimicrobial (Rukayadi and Hwang, 2006, 2013; Rukayadi et al., 2006, 2011), anti-inflammatory (Lim et al., 2005; Chung et al., 2007), antioxidant (Lim et al., 2005; Jantan et al., 2012), antihyperglycemic (Kim et al., 2014), antihypertensive (Ponce-Monter et al., 1999; Campos et al., 2000), antiplatelet (Jantan et al., 2008), nephroprotective (Kim et al., 2005), hepatoprotective (Kim et al., 2004; Hong et al., 2005), and estrogenic effect (Anggakusuma et al., 2009). Because of this property, it's important to explore *C. zanthorrhiza* accessions with high xanthorrhizol contents. The yield and biological activities of xanthorrhizol have previously been reported to be affected by geographical location (Nurcholis et al., 2012), but remains unclear whether it was caused by environmental factors or genetic variability. Furthermore, the evaluation of xanthorrhizol contents and pharmacological activities of the different accessions of *C. zanthorrhiza* remains unexplored, and knowledge is limited. α-Glucosidase inhibition and cytotoxic activities of Indonesia accessions have not been tested so far. This knowledge of accessions can develop further insight for *C. zanthorrhiza* breeders to finding new varieties.

* Corresponding author.
E-mail: wnurcholis@apps.ipb.ac.id (W. Nurcholis).

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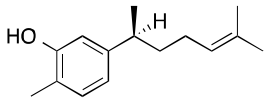
Therefore, in this study to examine yield in ethyl acetate fraction, xanthorrhizol contents, α-glucosidase inhibition, and cytotoxic activities characters without environmental influences, the rhizomes were grown under same environmental and soil conditions, so that the results are comparable and differences should reflect differences between the various accessions of *C. zanthorrhiza* genetically.



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Materials and methods

Plant material

The rhizome of four accessions and one variety of *Curcuma zanthorrhiza* Roxb., Zingiberaceae, in 2013 from different Indonesian locations were collected. Variety of *C. zanthorrhiza* namely Cursina-III that received from Indonesian Spices and Medicinal Crops Research Institute. The plant material was identified by Mr. Topik Ridwan, and voucher specimens have been deposited at Tropical Biopharmaca Research Center, Bogor Agricultural University (BMK2013080001-BMK2013080005). Sampling locations and their geographic coordinates are shown in Box 1. Rhizomes sample of *C. zanthorrhiza* were planted at the experimental site of SOHO Centre of Excellent in Herbal Research, Sukabumi, West Java, Indonesia (6°49′55.49″ S, 106°49′3.09″ E; average altitude of 1697 m) in October 2013. The cultivation was arranged in a completely randomized design with three replications. All rhizomes sample were grown under the same conditions with 50 cm × 60 cm spacing and fertilized with 20-ton manure ha^{-1} which given one month before planting. The rhizomes of plants were harvested at the nine months after planting (in June 2014). The rhizomes samples were cut, dried and powdered. The powder was stored at room temperature until the extraction.

Xanthorrhizol extraction

The extraction was performed by maceration method according to Hwang et al. (2000) with modification. Briefly, the powdered rhizomes (25 g) were extracted with 75% (v/v) methanol (250 ml) at room temperature for 24 h and then filtered using Whatman paper filter No. 4. The methanol extract was concentrated by evaporation (Buchi, R-250, Switzerland) at 50 °C. These extracts were then fractionated with water:ethyl acetate in a ratio of 1:1 (v/v). The ethyl acetate fraction was separated and then concentrated by rotary vacuum evaporator (Buchi, R-250, Switzerland) at 50 °C. These extracts of ethyl acetate fraction were recorded as yield and stored at 4 °C until analysis.

Xanthorrhizol analysis

The xanthorrhizol content in rhizomes sample of the ethyl acetate fraction was determined by HPLC using a xanthorrhizol standard which was isolated from the methanol extract of *C. zanthorrhiza* rhizome with purity 85.42% by HPLC analysis (Nurcholis et al., 2012). All solvents used were HPLC grade. Briefly, 50 mg of sample fraction was dissolved in 25 ml of ethanol by sonication for 1 h at room temperature. After filtration through a 0.45-μm membrane filter, an amount of 20 μl sample solutions were injected into HPLC system. HPLC analysis was performed using a system of LC-20A series (Shimadzu, Tokyo, Japan) with system equipped a diode array UV–vis detector. Chromatographic separation was achieved by using a Phenomenex C18 column (150 mm × 4.6 mm ID, 5 μm particle size) with column oven temperature at 40 °C. The mobile phase used consist of 0.001% formic acid in water (A) and methanol (B) with gradient elution program of 90–10% (A) for 0–12 min and 90% (A) for 13–17 min. Elution was carried out at flow rate 1 ml/min and monitored at 224 nm for quantitation of xanthorrhizol. Standard stock solutions of xanthorrhizol were prepared in methanol at concentrations of 200 μg/ml. Results were obtained by comparing with the standard of xanthorrhizol and then were expressed as a percentage (w/w) extract to weight basis.

α-Glucosidase inhibition analysis

The α-glucosidase inhibition of ethyl acetate fraction in samples was analyzed according to the method reported by Mayur et al. (2010). In brief, 10 μl sample of different concentrations was a mixture with 50 μl of 0.1 M phosphate buffer (pH 7.0), and 25 μl of 0.5 mM pNPG. This mixture reaction was added 25 μl of a α-glucosidase solution (0.2 Unit/ml) and incubated at 37 °C for 30 min. Before reading of the absorbance at 410 nm with a microplate reader (Epoch Biotech, USA), the enzymatic reaction was stopped by adding 100 μl of 200 mM Na_2CO_3. The inhibition activity was expressed as percentage inhibition of enzyme activity. The inhibition curves of α-glucosidase in different concentrations were prepared, and IC_{50} values were obtained.

Cytotoxic analysis

Screening of preliminary cytotoxic activity in ethyl acetate fraction of rhizomes sample (in the concentration of 10–200 μg/ml) was analyzed using the brine shrimp lethality test (BSLT) according to the general procedure described by Meyer et al. (1982). Cytotoxic activities were measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma–Aldrich, St. Louis, MO, U.S.A) colorimetric assay according to the method by Handayani et al. (2013) with some modification. MCF-7 cancer cell line (ATCC HTB 22) and Vero non-cancerous cell line (ATCC CCL 81) were used cytotoxic analysis, and there were obtained from Primate Research Center, Bogor Agricultural University. Cell lines were cultured in Dulbecco's minimum Eagle's medium (Gibco, Rockville, MD, U.S.A.) with supplemented with fetal bovine serum (10%; Sigma–Aldrich, St. Louis, MO, U.S.A), 100 μg/ml penicillin (Gibco, Rockville, MD, U.S.A.) and 100 μg/ml streptomycin (Gibco, Rockville, MD, U.S.A.). In brief, 2×10^{-3} cells/ml were exposed to different rhizomes sample concentration of 10–500 μg/ml in Vero cell and 3.75–60 μg/ml in MCF-7 cell for 72 h. The untreated cells as the control group were also included. After treatment, the medium was removed, and cells were incubated with 20 μl MTT (2 mg/ml). After 4 h incubation, 100 μl HCl-isopropanol (0.1 N) was added to the reaction mixture. Finally, the absorbance was measured with a microplate reader (Bio-Rad 680, USA) at 595 nm. The mortality percentage curves were prepared, and LC_{50} values were obtained.

Statistical analysis

All data were subjected to statistical analysis using Statistical Tool for Agricultural Research software version 2.0.1. Differences between the accessions were performed by Least Significant Difference (LSD) test. The yield in EA fraction, xanthorrhizol content, α-glucosidase inhibition, and cytotoxic activities traits of samples were used to determine the relationship between the different accessions of *C. zanthorrhiza* by cluster analysis using the Minitab 16 software. Euclidean distance was selected as a measure of similarity and the single linkage method was used for cluster dentition.

Box 1
Collection sites and geographical coordinates of *Curcuma zanthorrhiza* accessions sampled from different part of Indonesia.

Accession and variety	Region	Latitude (S)	Longitude (E)	Altitude (m)
Wonogiri	Wonogiri, Central Java, Indonesia	7°57′22.83″	110°59′37.51″	378
Karanganyar	Karanganyar, Solo, Central Java, Indonesia	7°39′49.37″	111°08′01.93″	1113
Ngawi	Ngawi, East Java, Indonesia	7°29′52.21″	111°09′22.78″	345
Sukabumi	Sukabumi, West Java, Indonesia	7°11′49.14″	106°30′40.58″	359
Cursina-III	Bogor, West Java, Indonesia	6°34′37.95″	106°47′20.37″	238

Results and discussion

There has been variation in phytochemical content and pharmacological activities of medicinal plant species that are growing in different geographical conditions (Nurcholis et al., 2016b,c; Wu et al., 2017). Therefore, the limit of environmental impact aims to encourage the plant selection for the breeding program better (Oliveira et al., 2013; Moghaddam and Pirbalouti, 2017). In this work, *C. zanthorrhiza* that collected from Indonesia were evaluated for characterization of yield in ethyl acetate fraction, xanthorrhizol content, α-glucosidase inhibition, and cytotoxic activities as well as found possible elite accessions without environmental effects. The phytochemical and pharmacological variation of *C. zanthorrhiza* has commercial importance as well as being helpful in the improvement for the food and pharmaceutical industries. All throughout reports of this discussion, four accessions and one variety of *C. zanthorrhiza* were evaluated for xanthorrhizol contents and pharmacological activities.

Yield in ethyl acetate fraction

Yield in ethyl acetate (EA) fraction of xanthorrhizol extraction from rhizomes sample are presented in Fig. 1. The EA fraction yield of different accessions of *C. zanthorrhiza* varied from 8.24 ± 0.88 (Karanganyar) to 13.13 ± 0.88% (w/w) (Sukabumi). No significant difference was observed in EA fraction yields between Cursina-III variety and the accessions of Ngawi and Sukabumi, but significant difference was shown with accessions of Wonogiri and Karanganyar in $p \leq 0.05$. Anggakusuma et al. (2009) revealed that EA fraction yield from *C. zanthorrhiza* rhizome collected from Jakarta, Indonesia was 4.8% (w/w). Musfiroh et al. (2013) reported that the EA fraction yield of *C. zanthorrhiza* rhizome collected from Lembang, West Java, Indonesia was 5.19%. The yield in EA fraction from all samples rhizome in this paper showed highest than previously reported ones.

Xanthorrhizol content

As seen in Fig. 2, xanthorrhizol contents in EA fraction of *C. zanthorrhiza* samples varied from 43.55 ± 0.35% to 47.99 ± 0.37% (w/w). Additionally, Wonogiri accession had significantly highest xanthorrhizol content compared with another accession (Karanganyar, Ngawi and Sukabumi) and the the control of Cursina-III variety in $p \leq 0.05$. Xanthorrhizol was a constituent of essential oil composition of *C. zanthorrhiza* (Zwaving and Bos, 1992). The previous study reported various ranges for the xanthorrhizol production in different *C. zanthorrhiza* accessions and environmental (Nurcholis et al., 2012). In some literature, essential oil production in the medicinal plant can be highly affected by both environmental factors and plant species (Oliveira et al., 2013; Moghaddam and Pirbalouti, 2017). Therefore, a variation of xanthorrhizol contents from different samples rhizomes in this study was affected by accessions genetic in the plant.

α-Glucosidase inhibition analysis

In the intestine human, inhibition of α-glucosidase was effective in delaying glucose absorption and preventing elevation of the postprandial blood glucose level; thus, α-glucosidase inhibitors used as a glycemic control in the treatment of diabetes (Sivasothy et al., 2016). This report investigated the inhibitory activities of four *C. zanthorrhiza* accessions against α-glucosidase and Cursina-III variety used as a control. The IC_{50} values of α-glucosidase inhibitory activities ranged from 339.05 ± 38.54 μg/ml to 455.01 ± 33.48 μg/ml (Fig. 3). The EA fraction from Karanganyar accession exhibited a higher α-glucosidase inhibitory activity (the lowest IC_{50} value) with significantly in $p \leq 0.05$, while the EA fraction from Ngawi accession showed the weakest activity (the highest IC_{50} value). All the rhizome samples had less α-glucosidase inhibitor activity with an IC_{50} value of >200 μg/ml. Therefore, the EA fraction of *C. zanthorrhiza* accessions and Cursina-III variety were not potential sources for α-glucosidase inhibitor active compounds.

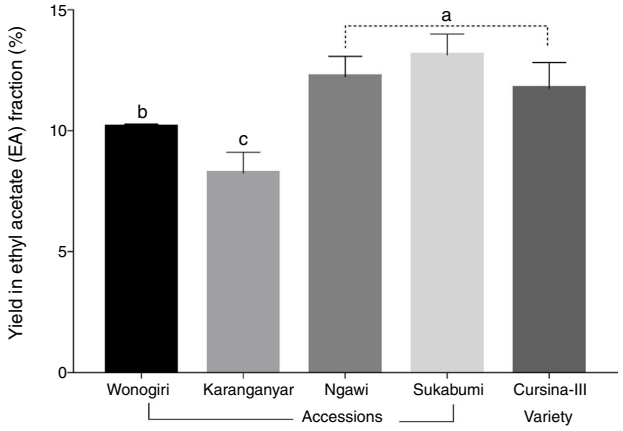

Fig. 1. Yield in ethyl acetate fraction of different accessions of *Curcuma zanthorrhiza* from Indonesia. Value followed by the same letters indicate they are not significantly different by LSD test at $p \leq 0.05$.

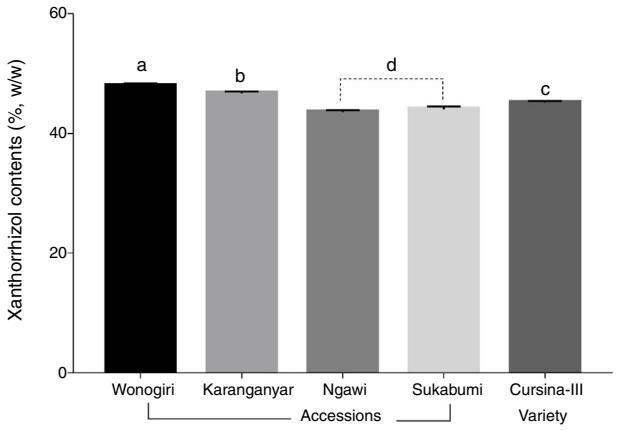

Fig. 2. Xanthorrhizol content in EA fraction of different accessions of *Curcuma zanthorrhiza* from Indonesia. Value followed by the same letters indicate they are not significantly different by LSD test at $p \leq 0.05$.

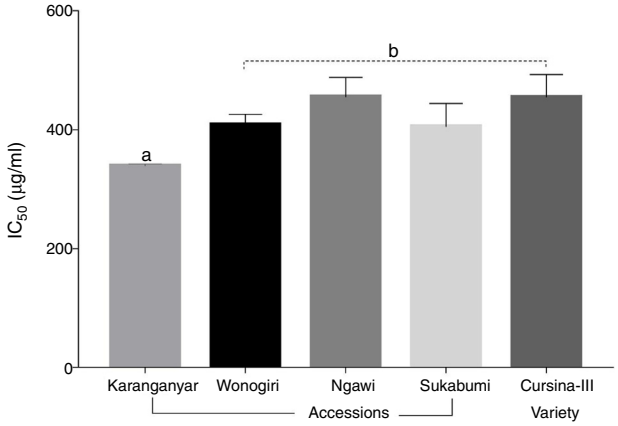

Fig. 3. α-Glucosidase inhibition in EA fraction of different accessions of *Curcuma zanthorrhiza* from Indonesia. Value followed by the same letters indicate they are not significantly different by LSD test at $p \leq 0.05$.

Opposite findings were reported by Awin et al. (2016), who evaluated in ethanol (50%; v/v) extract and found that *C. zanthorrhiza* extract, collected from Kuala Lumpur-Malaysia, had the highest α-glucosidase inhibition with IC_{50} of 36.6–73.1 μg/ml. Moreover, Hasimum et al. (2016) investigated the ethanol 96% extract of *C. zanthorrhiza* which collected from Bandung, West Java, Indonesia. Their results revealed that α-glucosidase inhibitory of *C. zanthorrhiza* was highest with an IC_{50} value of 78.9 μg/ml.

Cytotoxic activities

As shown in Fig. 4, the EA fractions of samples *C. zanthorrhiza* exhibited the highest cytotoxic activities, and all samples in cytotoxic activities were showed not significantly with the others in $p \leq 0.05$. The cytotoxicity of the EA fractions in different sample rhizomes of *C. zanthorrhiza* was evaluated using the BSLT for potency in preliminary screening for cytotoxins as anticancer. The LC_{50} values of BSLT ranged from 33.25 ± 7.99 μg/ml in EA fraction of Ngawi accession to 42.28 ± 11.35 μg/ml in EA fraction of Karanganyar accession (Fig. 4A). These results indicated that EA fraction in all samples were moderate toxic activities; because LC_{50} values were ranged of 10–100 μg/ml (Tanamatayarat, 2016). On Vero cell line, the LC_{50} values of EA fraction samples were recorded 3.10 ± 0.52 μg/ml (Karanganyar accession) to 9.85 ± 4.27 μg/ml (Cursina-III variety) (Fig. 4B). While on MCF-7 cell line, the LC_{50} values of EA fraction samples were ranged from 1.17 ± 0.83 μg/ml (Ngawi accession) to 6.83 ± 6.38 μg/ml (Sukabumi accession) (Fig. 4C). The result cytotoxic showed that of EA fraction in all samples rhizome of *C. zanthorrhiza* were potential sources for the anticancer application. Similar findings were reported by Cheah et al. (2006) that found the EC_{50} value of 1.71 μg/ml against MCF-7 cell line. Moreover, some researchers have been demonstrated antiproliferative activities of xanthorrhizol in many types of human breast cancer cells such as MDA-MB-231 with LC_{50} value of 8.67 μg/ml (Cheah et al., 2008), YMB-1 with LC_{50} value of 2.88 g/ml (Udin, 2013), and T47D with LC_{50} of 100 μg/ml (Musfiroh et al., 2013).

Hierarchical cluster analysis

To evaluate the apparent similarities and relationships among and within the *C. zanthorrhiza* accessions studied, hierarchical cluster analysis was performed based on Euclidean distances from yield in EA fraction, xanthorrhizol content, α-glucosidase inhibition, and cytotoxic activities data matrix. The result of hierarchical cluster analysis are showed in the form of a dendrogram in Fig. 5 and

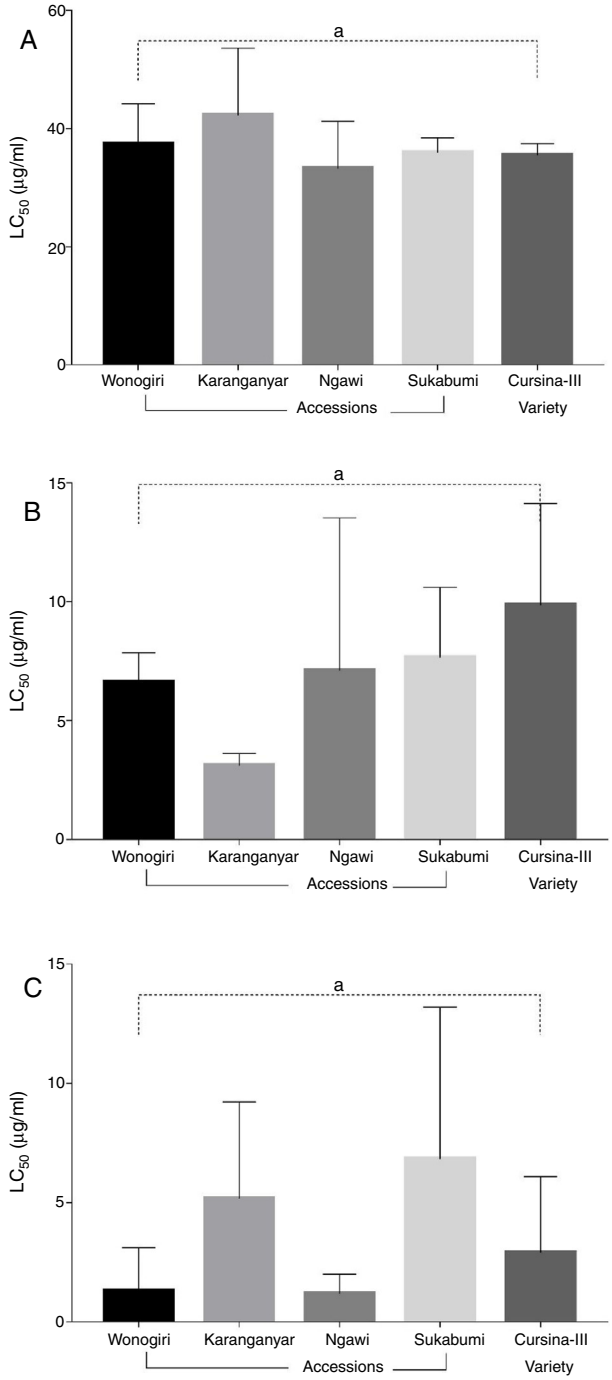

Fig. 4. Cytotoxicity in EA fraction of *Curcuma zanthorrhiza* accessions against brine shrimpt (A), Vero cell line (B), and MCF-7 cell line (C). Value followed by the same letters indicate they are not significantly different by LSD test at $p \leq 0.05$.

C. zanthorrhiza studied were classified into three groups on similarities of 49.67%. The first group consisted of Ngawi and Sukabumi accessions and Cursina-III variety. The second and third groups were contained one accession of Wonogiri and Karanganyar, respectively. *C. zanthorrhiza* samples studied were distinguished from the other group that was essential for high yield in EA fraction and xanthorrhizol characteristic; because in cytotoxicity and α-glucosidase inhibition of all samples had similarities that characterized by toxic active and less α-glucosidase inhibition, respectively. The *C. zanthorrhiza* samples of the first group had the highest yield of EA fraction (11.75–13.13%) followed by second group (10.15%) and the third group (8.24%). The

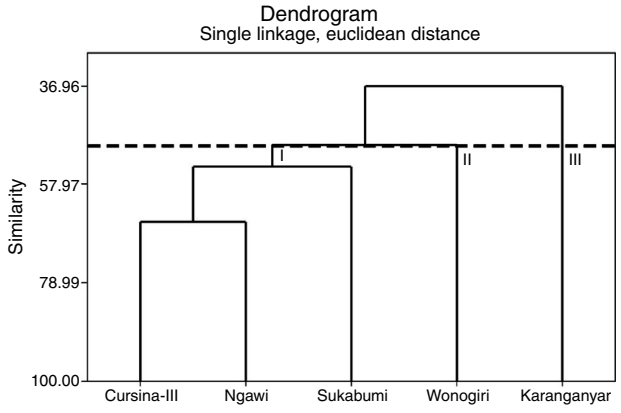

Fig. 5. Dendrogram showing the similarity among different accessions of *Curcuma zanthorrhiza* based on Euclidean distances from yield in EA fraction and xanthorrhizol content, α-Glucosidase inhibition, and cytotoxicity activities data matrix.

highest xanthorrhizol content was characterized in the second group (47.99%) and followed by the third group (46.73%) and first group (43.55–45.13%). This result indicated that accessions of Wonogiri and Karanganyar provide valuable information for selecting high-quality *C. zanthorrhiza* rhizome for the large-scale production of a xanthorrhizol compound in a breeding program for the pharmaceutical industry. Similarly, the previous study reported that Wonogiri accessions have highest of curcumin production and pharmacological activities (Nurcholis et al., 2016a).

Conclusion

In this work, yield in ethyl acetate fraction, xanthorrhizol content, α-glucosidase inhibition, and cytotoxic activities were characterized in four accessions and one variety of *C. zanthorrhiza*. In all samples studied had similarities pharmacological activities of cytotoxic activities and α-glucosidase inhibition that characterized by toxic active and less α-glucosidase inhibition, respectively. Wonogiri and Karanganyar accessions, compared with Cursina-III variety of *C. zanthorrhiza*, had highest xanthorrhizol contents and these accessions were provide the potential for high xanthorrhizol production in the breeding program on a commercial scale.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Author's contributions

WN contribution included collecting samples, designing laboratory work, analyzing the results, supervision of the laboratory work, and preparing the paper. AAM contribution included collecting samples and performing laboratory work. LA contribution included designing laboratory work, analyzing the results and preparing the paper. All the authors have read the final paper and approved the submission of the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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