ANTIBACTERIAL ACTIVITY OF GERMACRANE TYPE SESQUITERPENES FROM *Curcuma heyneana* RHIZOMES

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ABSTRACT

The isolation of terpenoids from C. heyneana rhizomes and their antibacterial activity have been conducted. The terpenoids were isolated by using vacuum liquid chromatography and radial chromatography. The structures of the compounds were determined based on spectroscopic data (^{1}H-NMR, ^{13}C-NMR (1D and 2D)). The antibacterial activity was carried out by using microdilution method and evaluated against eight bacteria. Three germacrane type sesquiterpenes have been isolated from C. heyneana rhizhomes and were identified as germacrone, dehydrocurdione, and 1(10),4(5)-diepoxygermacrone. Germacrone showed highest antibacterial activity against P. aeruginosa with MIC values of 15.6 µg/mL and MBC values 31.2 µg/mL. Dehydrocurdione showed highest antibacterial activity against B. subtilis with MIC values of 31.2 µg/mL and MBC values of 31.2 µg/mL. However, 1(10),4(5)-diepoxygermacrone showed a weak antibacterial activity.

Keywords: *C. heyneana; sesquiterpenes; antibacterial*

ABSTRAK

Telah dilakukan penelitian mengenai isolasi senyawa terpenoid dari rimpang C. heyneana dan uji aktivitas antibakterinya. Isolasi senyawa terpenoid dilakukan melalui teknik kromatografi cair vakum dan kromatografi radial. Struktur kimia senyawa hasil isolasi ditentukan berdasarkan data spektroskopi (^{1}H-NMR, ^{13}C-NMR (1D and 2D)). Uji aktivitas antibakteri dilakukan dengan metode mikrodilusi terhadap delapan spesies bakteri. Hasil isolasi dari rimpang C. heyneana diperoleh tiga senyawa seskuiterpen dengan kerangka germakran yaitu germakron, dehidrokurdion, and 1(10),4(5)-diepoksigermakron. Hasil uji aktivitas antibakteri diketahui bahwa germakron memiliki aktivitas tertinggi terhadap P. aeruginosa dengan nilai MIC 15,6 µg/mL dan MBC 31,2 µg/mL. Dehidrokurdion menunjukkan aktivitas tertinggi terhadap B. subtilis dengan nilai MIC dan MBC sama yaitu 31,2 µg/mL, sedangkan 1(10),4(5)-diepoksigermakron menunjukkan aktivitas antibakteri yang lemah.

Kata Kunci: *C. heyneana; seskuiterpen; antibakteri*

INTRODUCTION

Infections caused by multidrug-resistant bacteria are an increasing problem due to the emergence and propagation of microbial drug resistance and the lack of development of new antimicrobials [1]. Natural product has provided the pharmaceutical industry with some of its sources of lead products in the search for new antimicrobial [2]. From ancient time many medicinal plants represent as source of new antimicrobial agents [1,3]. The antimicrobial activities of plants oils and extract have formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies [4].

Curcuma heyneana is a member of the family Zingiberaceae. It is medicinal plants that widely cultivated in Java, Indonesia. In Indonesia, the rhizome of *C. heyneana* is called “temu giring”. It is used empirically as a traditional medicine for treat skin diseases, anthelmintic, hepatoprotective agent and as a menstruation promoter. Its crude extract is used to treat fatigue, obesity and rheumatism, and its fulverized form is used as a component in beauty treatments [5]. Phytochemically, previous investigation have been reported, that the major constituens of *C. heyneana* were the curcuminoids and sesquiterpenes [6]. More of

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twenty five the sesquiterpenoids have been isolated from rhizome of *C. heyneana* [5,7] and some biological activities have been reported, such as anti-inflammatory effects [8], cytotoxicity and antibacterial activities [9] and inhibitor of protein tyrosine phosphatase 1B [5].

In spite of its numerous medicinal properties and several compounds have been reported, the investigation of antimicrobial activity of sesquiterpenes from *C. heyneana* still limited. Previous investigation, reported that three sesquiterpenes (oxycurcumenol, curcumenol and isocurcumenol) from *C. heyneana* rhizomes have antibacterial activity using disc-diffusion methods [9]. Therefore, this study aimed to evaluate the antibacterial activity of others sesquiterpenes from *C. heyneana* rhizomes against eight common pathogenic bacteria by microdilution methods (MIC and MBC determination). The determination of MIC and MBC is regarded as a more precise evaluation of antimicrobial property, since those determinations are more sensitive than the disc-diffusion assay [4].

There is an urgent need to search for new antimicrobial compounds with diverse chemical structures and novel mechanism of action because there has been an alarming increase in the incidence of infection diseases, as well as the development of resistance to antibiotic in current clinical uses [4]. In addition, the properties of medicinal plants for natural preservative need to prevent microbial spoilage and therefore to prolong the shelf life of the food, and finally to protect the consumers from infection disease.

EXPERIMENTAL SECTION

Materials

The rhizomes of *C. heyneana* were collected from Solo, Indonesia. Eight strains of bacteria was used in study, which include two Gram-positive bacteria (*B. subtilis* and *S. aureus*) and six Gram-negative bacteria (*E. aerogenes*, *E. coli, P. aeruginosa*, *Salmonella typhii*, *Shigella dysentriae* and *Vibrio cholerae*). These bacterial strains were isolated from clinical samples and obtained from Microbiology Laboratory, Politeknik Kesehatan Bandung, Indonesia.

Instrumentation

^{1}H- and ^{13}C-NMR (1D and 2D) spectra were performed on Agilent DD2 system operating at 500 (^{1}H) MHz and 125 (^{13}C) MHz. Optical rotations was measured by Rudolf Research Analytical Autopol IV Auto Polarimeter. Antibacterial activity was determined by Bio-Rad xMark Microplate Spectrophotometer.

Procedure

Extraction and isolation of pure compounds

Powder of *C. heyneana* rhizomes (1 kg) was extracted with acetone (10 L, 3 times) for 3 days at room temperature, then filtrated and evaporated to give acetone extract. The acetone extracts was partitioned with *n*-hexane and methanol respectively, to give *n*-hexane soluble fraction and methanol soluble fractions. *n*-Hexane soluble fractions (20 g) was fractionated by using silica gel column (vacuum liquid chromatography) and eluted step-wise with 200 mL *n*-hexane, 200 mL *n*-hexane:chloroform (9:1, 8:2, 7:3, 6:4, 3:7), 200 mL chloroform, 200 mL ethylacetate and 200 mL methanol, respectively. All fractions were concentrated on rotary evaporator, then loaded on TLC plate and fractions having similar R_f values were pooled sub-fractions, afforded fractions A: 366 mg, B: 2040 mg, C: 1545 mg, D: 2755 mg, and E:4043 mg. Fractions B was purified by radial chromatography and eluted with *n*-hexane : chloroform (19:1) yield compound 1 (23 mg). Fractions C was purified by radial chromatography and eluted with *n*-hexane : chloroform (7:3) yield compound 2 (16 mg). Then Fractions E was purified by radial chromatography and eluted with *n*-hexane : ethyl acetate (1:1) yield compound 3 (32 mg). The molecule structures of compounds 1, 2 and 3 were identified by NMR (1D and 2D) spectrometer and polarimeter, and then compared with the literature data.

Antibacterial activity assays

Determination of antibacterial activity using the broth microdilution methods, according to the methods suggested by the Clinical and Laboratory Standards Institute [10].

Preparation of bacterial suspension. The bacteria colonies from the fresh agar plate transferred into a sterile capped glass tube containing sterile broth or saline solution (NaCl 0.9 % b/v) using a sterile loop or cotton swab and mixed well. The suspension adjusted to achieve a turbidity equivalent to a 0.5 Mc Farland turbidity standard.

Determination of minimum inhibitory concentration (MIC). The samples were dissolved in dimethyl sulfoxide (DMSO). The samples were prepared to achieve 250 µg/mL for isolated compounds in the first well. Two-fold dilution of samples was performed in 96-wells microplate over the range 1.95 to 250 µg/mL. This was achieved by filling all wells with 200 µL of Mueller Hinton Broth (MHB) medium. Then 200 µL of samples was transferred into the first well. Two-fold serial dilution was performed by transfer 200 µL of the

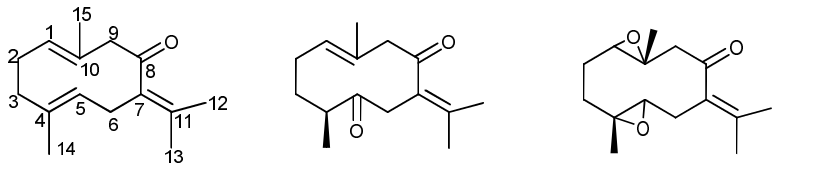

Fig 1. Structures of the isolated compounds

Table 1. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz) spectral data of the isolated compounds [in $CDCl_3$]

C	Germacrone		Dehydrocurdione		1(10),4(5)-Diepoxygermacrone	
	δ ^{1}H (*mult*) ppm	δ ^{13}C ppm	δ ^{1}H (*mult*) ppm	δ ^{13}C ppm	δ ^{1}H (*mult*) ppm	δ ^{13}C ppm
1	4.99 (*d*)	132.70	5.11 (*t*)	128.95	2.01 (*d*)	56.08
2	2.16 (*m*)	38.07	2.08 (*m*)	22.37	1.67 (*m*)	22.05
3	2.11 (*m*)	24.08	1.61-2.04 (*m*)	30.16	1.67 (*m*)	39.45
4	-	126.65	2.36 (*t*)	42.56	-	78.85
5	4.71 (*d*)	125.37	-	207.10	1.38 (*t*)	51.90
6	2.95 (*d*); 3.42(*d*)	29.24	3.24 (*dd*)	39.41	1.98 (*d*); 2.87 (*d*)	28.49
7	-	129.50	-	132.99	-	135.66
8	-	207.97	-	203.08	-	202.24
9	2.95 (*s*)	55.91	3.02-3.19 (*dd*)	52.96	2.48 (*d*); 2.95 (*d*)	60.18
10	-	135.02	-	125.31	-	71.47
11	-	137.30	-	125.88	-	139.13
12	1.73 (*s*)	21.99	1.71 (*s*)	16.99	1.87 (*s*)	21.04
13	1.78 (*s*)	19.91	1.74 (*s*)	18.09	1.81 (*s*)	21.82
14	1.44 (*s*)	15.59	0.98 (*d*)	14.37	1.16 (*s*)	21.89
15	1.63 (*s*)	17.11	1.60 (*s*)	12.26	1.10 (*s*)	19.70

mixture in the first well into the next consecutive well until the end of the row. At the last well 200 µL of the mixture was discharged, so that the total volume solution in each well was 200 µL. Then 10 µL bacterial suspension was transferred into all wells. The final test concentration of bacteria will be approximately 5 x 10^5 CFU/mL. The microplate was incubated for 24 h at 37 °C. Bacterial growth was determined using a universal microplate reader. MIC is defined as the lowest concentration at which no visible bacterial growth was observed. The positive controls used in this assays were chloramphenicol.

Determination of minimum bactericidal concentration (MBC). To determine MBC, aliquot removed from wells that showed no bacterial growth were streaked onto MHA plate and incubated under the same conditions. MBC was defined as the lowest concentration at which colonies failed to grow after incubated.

RESULT AND DISCUSSION

Isolation and Identifications of Isolated Compounds

Extraction of 1 kg of *C. heyneana* rhizomes powder yielded 91 g extract. The acetone extracts was partitioned with *n*-hexane and methanol respectively, yielded 35 g *n*-hexane soluble fraction and 38 g methanol soluble fractions. Repeated column chromatography and radial chromatography of *n*-hexane soluble fractions of *C. heyneana* rhizomes (20 g) resulted in the isolation of three sesquiterpenes. The isolated compounds were germacrane type sesquiterpenes and the structures of compound 1 was identical to germacrone (23 mg), compound 2 was identical to dehydrocurdione (16 mg), and compound 3 was identical to 1(10),4(5)-diepoxy-germacrone (32 mg). Previously, germacrone, dehydrocurdione and 1(10),4(5)-diepoxygermacrone (Fig. 1) have been isolated from the others species of *Curcuma* [6].

Germacrone was obtained as white crystal. The ^{1}H-NMR ($CDCl_3$, ppm) spectrum (Table 1) indicated the presence of four methyl protons (δ: 1.44 *s*, 1.63 *s*, 1.73 *s*, 1.78 *s*), four methylene protons (δ: 2.11 *m*, 2.16 *m*, 2.95-3.42 *dd*, 2.95 *s*) and two vinylic proton (δ: 4.99 *d,* 4.72 *d*). The ^{13}C-NMR spectrum (Table 1) contained 15 carbons (ppm), four methyl carbons (δ: 15.59, 17.11, 19.91, 21.99), four methylene carbons (δ: 24.08, 29.71, 38.07, 55.91), two vinylic carbon (δ: 125.37, 132.70), four quaternary carbons (δ: 126.65, 129.09, 135.02, 137.30) and a carbonyl carbon (δ; 207.97). The NMR spectra data of germacrone was consistent with published data [11].

Table 2. Antibacterial activity (µg/mL) of the isolated compounds

Bacteria	Germacrone		Dehydro-curdione		1(10),4(5)-Diepoxy-germacrone		Chloram-phenicol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. subtilis	125	125	31.2	31.2	125	250	1.9	1.9
S. aureus	62.5	62.5	250	250	250	250	1.9	1.9
E. aerogenese	125	125	125	125	125	125	3.9	3.9
E. coli	62.5	62.5	62.5	62.5	125	125	1.9	1.9
P. aeruginosa	15.6	31.2	125	125	125	125	31.2	31.2
S. typhii	62.5	62.5	250	250	125	125	3.9	3.9
S. dysentriae	125	125	125	125	125	125	1.9	1.9
V. cholerae	62.5	62.5	125	125	125	125	15.6	15.6

Dehydrocurdione was obtained as colorless oil, its optical rotation $[\alpha]_D$ was +68° in $CHCl_3$. The ^{1}H-NMR ($CDCl_3$, ppm) spectrum (Table 1) indicated the presence of four methyl protons (δ: 0.98 *d*, 1.60 *s*, 1.71 *s*, 1.74 *s*), four methylene protons (δ: 1.61-2.04 *m*, 2.08 *m,* 3.24 *dd*, 3.02-3.19 *dd*) a methine protons (δ: 2.36 *t*) and a vinylic proton (δ: 5.11 *t*). The ^{13}C-NMR spectrum (Table 1) contained 15 carbons (ppm), four methyl carbons (δ: 12.26, 14.37, 16.99, 18.09), four methylene carbons (δ: 26.34, 34.15, 43.39, 56.96), a methine carbon (δ: 46.59), a vinylic carbon (δ: 132.95), three quaternary carbons (δ: 125.31, 125.88, 132.99) and two carbonyl carbons (δ; 203.08; 207.10). The ^{1}H-NMR spectra data of dehydrocurdione was newest data from published data [12].

1(10),4(5)-Diepoxygermacrone was obtained as solid oil, its optical rotation $[\alpha]_D$ was -50° in $CHCl_3$. The ^{1}H-NMR ($CDCl_3$, ppm) spectrum (Table 1) indicated the presence of four methyl protons (δ: 1.10 *s*, 1.16 *s*, 1.81 *s*, 1.87 *s*), four methylene protons (δ: 1.67 *m*, 1.67 *t,* 1.98-2.87 *dd*, 2.48-2.95 *dd*), and two methine protons (δ: 1.38 *t,* 2.01 *t*). The ^{13}C-NMR spectrum (Table 1) contained 15 carbons (ppm), four methyl carbons (δ: 19.70, 21.04, 21.82, 21.89), four methylene carbons (δ: 22.05, 28.49, 39.45, 60.18), two methine carbons (δ: 51.90, 56.08), four quaternary carbons (δ: 71.47, 78.85, 135.66, 139.13) and a carbonyl carbon (δ: 202.24). The NMR spectra data of these compounds was newest data from published data, and based on its optical rotation though to be the stereoisomer of which have been reported [13-14].

Antibacterial Activity

MIC and MBC results (Table 2) indicate that the isolated compounds had different levels of activity against the bacteria. The inhibitory properties and bactericidal of isolated compounds were observed within range 1.9 to 250 µg/mL.

Based on the results, it showed that highest activity of germacrone was observed against *P. aeruginosa*, which had the lowest MIC and MBC value of 15.6 and 31.2 µg/mL respectively, followed by *S. aureus, E. coli*, *S. typhii* and *V. cholera*e (MIC and MBC 62,5 µg/mL), then *E. aerogenese* and *S. dysentriae* (MIC and MBC 125 µg/mL). In addition, germacrone showed the MIC value less than chloramphenicol (MIC 31.2 µg/mL) against *P. aeruginosa*, but the MBC value of germacrone and chloramfenicol was the same. It is suggest that germacrone was more capable to inhibit the growth of *P. aeruginosa* than chloramphenicol, but both of them were bactericidal at the same concentration.

Highest activity of dehydrocurdione was observed against *B. subtilis* which had the lowest MIC and MBC value of 31.2 µg/mL, which suggest that dehydrocurdion is both inhibitory and bactericidal at a single concentration. Followed by *E. coli* (MIC and MBC 62.5 µg/mL), then *E. aerogenese*, *P. aeruginosa*, *S. dysentriae*, *V. cholera*e (MIC and MBC 125 µg/mL), and *S. aureus* and *S. typhii* (MIC and MBC 250 µg/mL). Meanwhile, 1(10),4(5)-diepoxygermacrone showed a weak activity against all tested bacteria, which had MIC and MBC within range 125-250 µg/mL.

The differences in the antibacterial activity of isolated compounds might be due concentration, chemical structure and functional groups of the compounds, and the species of microorganism used. The varying degrees of sensitivity of the bacterial test organism may be due to both the intrinsic tolerance of microorganism and physical and chemical characteristics of the compounds [15].

The mode of action of several terpenoids has been studied, but actual structure-activity relationships of the terpenoids are not well understood. Investigations have shown that the site of action of cyclic hydrocarbon, including terpene hydrocarbons is at the cell membrane. Terpenoid compounds were shown to permeabilize the membranes, making them swell. This inhibits respiratory enzymes, which are crucial to the energy system in a cell [16]. The impairment of microbial activity by the terpenoids most likely result from hydrophobic interaction with the

membrane, which affects the functioning of the membrane and membrane-embedded proteins [17].

The results of this study suggest that the constituents of *C. heyneana* rhizomes have potent antibacterial properties. However, further phytochemical and pharmacological are necessary to investigate the bioactive compounds and study their mechanism of action.

CONCLUSION

Three germacrane type sesquiterpenes have been isolated from *C. heyneana* rhizomes and were identified as germacrone, dehydrocurdione, and 1(10),4(5)-diepoxygermacrone. Germacrone showed highest antibacterial activity against *P. aeruginosa* with MIC values of 15.6 µg/mL and MBC values 31.2 µg/mL. Dehydrocurdione showed highest antibacterial activity against *B. subtilis* with MIC values of 31.2 µg/mL and MBC values of 31.2 µg/mL. However, 1(10),4(5)-diepoxygermacrone showed a weak antibacterial activity.

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