Antibacterial Activity of Benzyl Benzoate and Crotepoxide from *Kaempferia rotunda* L. Rhizome

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Abstract: *Benzyl benzoate and crotepoxide are the major components of* Kaempferia rotunda *L. rhizome. However, the bioactivity study of benzyl benzoate and crotepoxide as the antibacterial activity were still limited. Therefore, the antibacterial activity of benzyl benzoate and crotepoxide against four pathogenic bacteria, i.e.,* Escherichia coli *ATCC 25922,* Enterococcus aerogenes *ATCC 13048,* Bacillus cereus *ATCC 6538 and* Staphylococcus aureus *ATCC 11778 were investigated. The isolation steps included the extraction by maceration with acetone, and then the acetone extract was partitioned with* n-*hexane:methanol (1:1) and ethyl acetate:water (1:1) respectively. The isolation by liquid vacuum chromatography followed by column chromatography was yielded benzyl benzoate from the* n-*hexane fraction and crotepoxide from ethyl acetate fraction. The molecular structure of isolated compounds was identified based on NMR (1D and 2D) spectroscopic data. The antibacterial activity assay of isolated compounds was carried out using the disc diffusion method. The antibacterial evaluation confirms that the benzyl benzoate and crotepoxide exhibits a medium level activity. Benzyl benzoate showed highest antibacterial activity against* B. cereus *with MIC of 50 µg/mL and inhibitory zone of 5.9 mm, while the crotepoxide showed highest antibacterial activity against* E. aerogenes *with MIC of 100 µg/mL with inhibitory zone 6.1 mm.*

Keywords: *antibacterial; benzyl benzoate; crotepoxide;* K. rotunda *L.*

■ INTRODUCTION

Infectious disease is one of the health problems, especially in developing countries including in Indonesia. Treatment of infectious diseases by bacteria with antibiotics has been carried out, but the ability of antibiotics gradually decreased due to the resistance of microorganism. In addition, the use of synthetic antibiotics often causes adverse effects. It encourages the researchers to get the new safe antibiotics, one of them from medicinal plants.

Kaempferia rotunda (Zingiberaceae) is a medicinal plant in Indonesia; It was known locally name as "*kunci pepet*" or "*kunir putih*". The rhizome of *K. rotunda* was used for traditional medicine such as treating stomach pain, fever, indigestion, inflammation due to bruises or sprains, carminative and accelerate wound healing [1]. The crude extracts, volatile oils and isolated compounds from *K. rotunda* rhizome exhibited the essential biological activities. According to the previous report, extract of *K. rotunda* rhizome showed antioxidant activity [2-3], insecticides [4], anti-inflammatory [5], anthelmintic [6], antihyperglycemic and antinociceptive [7], antimicrobial [8-10] and anti-androgenic [11]. Some compounds of the *K. rotunda* rhizome also revealed some biological activities. A 2-hydroxy-4,4',6-trimethoxy chalcone showed antioxidant activity with IC_{50} of 142 µg/mL [3]. Crotepoxide was the main constituent of *K. rotunda* rhizome useful for antitumor agent [5]. In the ethyl acetate and ethanol extract of *K. rotunda* were contain 33.11 and 42.92% crotepoxide, respectively [11]. Pinostrobin, 5,7-dihydroflavanone, and crotepoxide were exhibited anticancer activity against T470 breast cancer cell with IC_{50} of 59.8, 122.71 and > 1000 µg/mL, respectively [12]. Meanwhile, benzyl

benzoate showed insecticidal activity on *Spodoptera littoralis* with LC_{50} of 5.6 µg/mL [4].

The essential oil has an important role in the biological activity of *K. rotunda,* The essential oil of *K. rotunda* rhizome was contained about 75 compounds with two main compounds namely benzyl benzoate (69.7%) and *n*-pentadecane (22.9%) [13]. In different locations, it was also mentioned that of 20 compounds in the volatile oil of *K. rotunda* was contain benzyl benzoate 36.60% and bornyl acetate 30.15% [14]. Furthermore, it was reported that essential oils in *n*-hexane extract of *K. rotunda* rhizome could inhibit the growth of some bacteria [9]. The other plant study reported that *Salvia urmiensis* essential oil contained 60.3% benzyl benzoate showed high activity against *Staphylococcus epidermis* and *Staphylococcus cerevisiae* with minimum inhibitory of 9.3 µg/mL [15]. It showed that benzyl benzoate has potential as an antibacterial agent because it is the main component of *K. rotunda* essential oil.

The previous study of some extracts of *K. rotunda* rhizome suggests that the ethyl acetate and water extracts showed significant antibacterial activity against some pathogenic bacteria, whereas the antibacterial activity of benzyl benzoate and crotepoxide were still limited reported. In this article, we wish to report the isolation of the major component of *K. rotunda* rhizome as well as antibacterial properties.

■ EXPERIMENTAL SECTION

Materials

Rhizome of K. *rotunda* (collected from Purwokerto Indonesia), silica gel plate 60 F_{254} aluminium sheets (Merck), silica gel 60 G (7731, 7734 and 7733, Merck), bacterial strains: *E. coli* ATCC 25922, *E. aerogenes* ATCC 13048, *B. cereus* ATCC 6538 and *S. aureus* ATCC 11778 (supplied by Microbiology Laboratory, Faculty of Medicine Unsoed Purwokerto), Muller Hinton Agar (Oxoid), chloramphenicol (Merck) and dimethyl sulfoxide (Merck).

Instrumentation

^{1}H and ^{13}C-NMR (Nuclear Magnetic Resonance) spectra used Agilent DD2 spectrometer operating at 500 (^{1}H) and 125 (^{13}C) Mhz. Optical rotation was measured by Rudolf Research Analytical Autopol IV Auto Polarimeter.

Procedure

Isolation of benzyl benzoate and crotepoxide from K. rotunda

Dried powder of *K. rotunda* rhizome (1 kg) was extracted with acetone at room temperature. The acetone extract of *K. rotunda* rhizome filtered and concentrated using a rotary evaporator. Then the concentrated acetone extract was partitioned with *n*-hexane:methanol (1:1) and the soluble *n*-hexane extract was concentrated with a rotary evaporator. On the other hand, the soluble methanol extract was partitioned with ethyl acetate:water (1:1). Next, the ethyl acetate fraction was concentrated with a rotary evaporator.

The *n*-hexane fraction of *K. rotunda* rhizome (20 g) was fractionated by vacuum column chromatography on silica gel and eluted gradually with *n*-hexane, the mixture of *n*-hexane:chloroform (7:3, 6:4, 5:5, 2:8 and 1:9), chloroform, and ethyl acetate. TLC analysis was carried out to all fractions with eluent *n*-hexane:chloroform (1:1). The fractions having similar spot were collected into 7 sub-fractions: F1 (9.5 g), F2 (1.3 g), F3 (0.6 g), F4 (0.2 g), F5 (0.3 g), F6 (0.3 g) and F7 (0.5 g). Furthermore, F1 which contains the main components of benzyl benzoate was purified by column chromatography using eluent *n*-hexane:chloroform (9:1) to yield a pure benzyl benzoate in the form of colorless oil (543 mg). The ethyl acetate fraction of *K. rotunda* rhizome (6 g) was fractionated by vacuum column chromatography then eluted gradually with *n*-hexane: ethyl acetate (8:2, 7.5:2.5, 7:3 and 0:10) to give 5 sub-fraction: F1' (0.05 g), F2' (0.1 g), F3' (0.3 g), F4' (0.9 g), and F5' (1.3 g). Crotepoxide (colorless needles) was isolated from fraction F5' by column chromatography using eluent *n*-hexane:chloroform:ethyl acetate (5:5:1).

Antibacterial activity assays [16]

Selected bacteria were cultured for 24 h at 37 °C under aerobic conditions on agar media (Mueller Hinton Agar). Afterward, the bacteria were suspended in a 0.9% NaCl solution (w/v). The turbidity of

suspension of bacteria was corrected to the 0.5 Mc Farland standard (1-2 × 10^8 bacterial cells /mL).

Agar plate was inoculated with 200 µL bacterial suspension. 50 µL of the samples with concentrations of 10, 50, 100 and 500 µg/mL were dripped on paper disc on agar media and incubated for 24 h at 37 °C. The presence of clear zones around the paper disc was indicated that the sample has antibacterial activity. The inhibitory zone of the sample was determined by measuring the diameter of the clear zone around the paper disc. The assays were also carried out to the negative control (DMSO 10%) and standard antibiotic chloramphenicol (positive control). The assays were conducted in three repetitions.

■ RESULTS AND DISCUSSION

Identification of Benzyl Benzoate and Crotepoxide from *K. rotunda* Rhizome

Benzyl benzoate (Fig. 1) was obtained as colorless oil. The ^{1}H-NMR spectrum (500 MHz, $CDCl_3$) of benzyl benzoate was indicated seven proton signals. They revealed an oxygenated methylene signal at δ 5.39, (2H, *s*), and six signals of two aromatic proton that represented 10H which are at δ 7.36 (2H, *t*, J = 7.2 Hz, H-3, H-5), δ 7.41 (2H, *t*, J = 7.2 Hz, H-3' and H-5'), δ 7.44 (1H, *t*, J = 7.5 Hz, H-4'), δ 7.47 (2H, *d*, J = 7.2 Hz, H-2', H-6'), δ 7.58 (1H, *t*, J = 7.2 Hz, H-4), and δ 8.11 (2H, *d*, J = 7.5 Hz, H-2, H-6) ppm. Three proton triplet (H-3, H-5, and H-4 or H-3', H-5' and H-4') and two proton doublet (H-2, H-6 or H-2', H-6') could be assigned to phenyl groups which indicates a substituent on an aromatic ring. The ^{13}C-NMR spectrum (125 MHz, $CDCl_3$) confirmed that there are 14 carbon signals, which indicated the presence of two sp^3-carbon of oxygenated methylene (CH_2-O-) at δ 66.69 (C-8) ppm, a sp^2-carbon of carbonyl ester group at δ 166.43 (C-7) ppm, two quaternary sp^2-carbons at δ 130.15 (C-1) and 136.07 (C-1') ppm, and ten sp^2-methines at δ 129.70 (C-2, C-6), δ 128.16 (C-3, C-5), δ 133.02 (C-4), δ 128.37 (C-2', C-6'), δ 128.60 (C-3', C-5') and δ 128.24 (C-4') ppm. The ^{1}H and ^{13}C-NMR spectra data of benzyl benzoate (Table 1) was the newest data from published data [17], where previous data was operating at 300 (^{1}H) and 75 (^{13}C) Mhz.

Crotepoxide (Fig. 1) was obtained as colorless needles (mp 152–154 °C, $[\alpha]_D^{22}$ +66°). The ^{1}H NMR (500 MHz, $CDCl_3$) spectrum showed two signal for two methyl of acetyl groups at δ 1.96 (3H, *s*, H-11) and δ 2.05 (3H, *s*, H-12) ppm, five signal of the aromatic ring at δ 7.96 (2H,

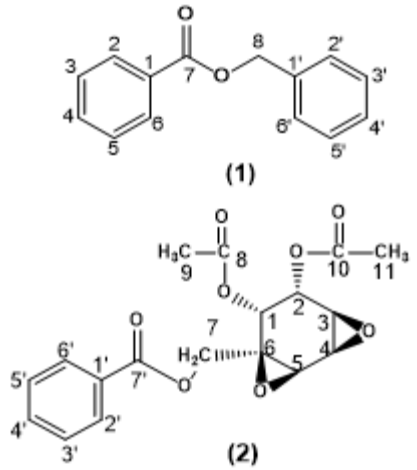

Fig 1. Benzyl benzoate (1) and Crotepoxide (2)

Table 1. HSQC and HMBC spectra of benzyl benzoate

C atom	HSQC		HMBC (^{1}H→^{13}C)
	δ_C ppm	δ_H (*mult*, *J* Hz) ppm	
1	130.15	-	-
2, 6	129.70	8.11 (2H, *d*, 7,2)	C-1, C-3, C-4, C-5, C-7
3, 5	128.16	7.36 (2H, *t*, 7,2)	C-2, C-4, C-7
4	133.02	7.58 (1H, *t*, 7,5)	C-2, C-3, C-5, C-6
7	166.43	-	-
8	66.69	5.39 (2H, *s*)	C-7, C-1', C-2'
1'	136.07	-	-
2', 6'	128.37	7.47 (2H, *d*, 7,2)	C-8, C-1', C-3', C-4'
3', 5'	128.60	7.41 (2H, *t*, 7,2)	C-1', C-2', C-4'
4'	128.24	7.44 (1H, *t*, 7,5)	C-2', C-3', C-5', C-6'

Table 2. HSQC and HMBC spectra of crotepoxide

C atom	HSQC		HMBC (^{1}H→^{13}C)
	δ_C ppm	δ_H (*mult*, *J* Hz) ppm	
1	59.39	-	-
2	69.40	5.64 (1H, *d*, 9.0)	C-1, C-3
3	70.36	4.91 (1H, *d*, 9,2)	C-2
4	52.61	3.03 (1H, *d*, 5.0)	C-3, C-6
5	48.07	3.39 (1H, *dd*, 5.0 and 2.7)	C-6
6	53.82	3.60 (1H, *d*, 2.7)	C-5
7	62.40	4.50 (1H, *d*, 12)	C-7'
		4.17(1H, *d*, 12)	
8	169.76	-	-
9	20.65	1.96 (3H, s)	-
10	170.06	-	-
11	20.68	2.05 (3H, s)	C-10
1'	129.08	-	-
2', 6'	129.79	7.96(2H, *dd*)	C-1', C-3', C-5', C-7'
3', 5'	128.56	7.39 (2H, *t*)	C-4', C-2', C-6'
4'	133.56	7.53 (1H, *t*)	C-2', C-3', C-5', C-6'
7'	165.78	-	-

m, H-2', H-6'), δ 7.39 (2H, *m*, H-3', H-5') and δ 7.53 (1H, *m*, H-4') ppm, three signal for oxygenated protons δ 3.39 (1H, *dd*, J = 2.5 and 3.9 Hz, H4), δ 3.08 (1H, *dd*, J = 0.8 and 3.5 Hz, H5) and δ 3.60 (1H, d, J = 2.7 Hz, H6) ppm, two signal for oxygenated protons at δ 5.64 (1H, d, 9.0 Hz) and δ 4.91 (1H, d, 9.0 Hz) ppm, and two signal for AB system at δ 4.50 (1H, J = 12.0 Hz, H-7) and 4.17 (1H, J = 12.0 Hz, H-7) ppm. The ^{13}C-NMR spectra (125 MHz, $CDCl_3$) of crotepoxide revealed the presence two of methyl of acetyl carbons at δ 20.65 (C-9) and 20.68 (C-11) ppm, six aromatic carbon at δ 129.08 (C-1'), 129.79 (C-2' and C-6'), 128.56 (C-3' and C-5'), and 133.56 (C-4') ppm, a methylene carbon at 62.40 (C-7) ppm, three carbonyl esters at δ 169.76 (C-8), 170.06 (C-10) and 165.78 (C-7') ppm, five methine carbons at δ 60.40 (C-2), 70.36 (C-3), 52.51 (C-4), 43.07 (C-5) and 53.82 (C-6) ppm, and two quaternary carbons at δ 59.39 (C-1) and 129.08 (C-1') ppm. The ^{1}H and ^{13}C-NMR spectra data of crotepoxide was in agreement with the published data [18].

The one bond correlation between carbon and proton is determined by the 2D (two-dimensional) NMR spectrum of HSQC (Heteronuclear Single Quantum Coherence), whereas the correlation of two or three bonds between carbon and proton is determined by the HMBC (Heteronuclear Multiple Bond Coherence) spectra. Table 1 and 2 represents the 1D (^{1}H and ^{13}C) and 2D (HSQC and HMBC) of benzyl benzoate and cretopoxide spectra data. Meanwhile, Fig. 2 describes the correlation of two or three bonds between proton and carbon (HMBC) on the benzyl benzoate and crotepoxide structures.

Antibacterial Activity

Antibacterial activity assays of extract and isolated compounds were conducted on four pathogenic bacteria including two Gram-negative bacteria *E. coli* ATCC 25922

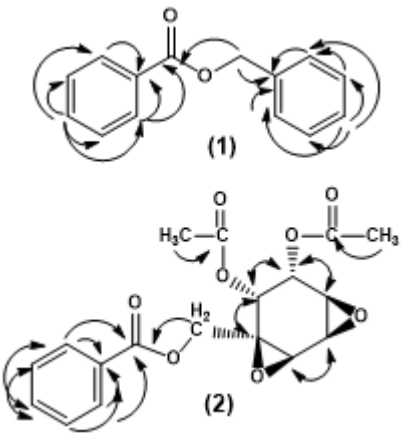

Fig 2. HMBC of benzyl benzoate (1) and crotepoxide (2)

Table 3. Antibacterial activity of extract, fraction and isolated compounds of *K. rotunda* rhizome

Sample	Concentration (μg/mL)	Zone inhibitory (mm)±SD			
		E. coli ATCC 25922	*E. aerogenes* ATCC 13048	*B. cereus* ATCC 6538	*S. aureus* ATCC 11778
Acetone extract	10	-	-	-	3.1±0.06
	50	3.4±0.03	-	-	5.4±0.26
	100	8.0±0.03	4.3±0.17	3.6±0.06	8.1±0.17
	500	11.7±0.17	7.9±0.02	10.1±0.36	12.1±0.36
n-Hexane fraction	10	4.2±0.11	3.0±0.06	4.1±0.03	4.0±0.06
	50	8.2±0.06	8.1±0.11	7.9±0.17	10.0±0.03
	100	11.1±0.36	12.2±0.86	15.9±0.01	15.2±0.26
	500	20.2±0.06	18.9±0.03	19.8±0.66	19.5±0.50
Ethyl acetate fraction	10	-	-	-	-
	50	-	6.9±0.11	-	-
	100	-	9.5±0.01	-	2.4±0.26
	500	5.0±0.03	11.8±0.36	3.6±0.36	4.1±0.17
Benzyl benzoate	10	-	-	-	-
	50	-	-	5.9±0.06	3.6±0.06
	100	5.2±0.17	4.0±0.06	8.1±0.06	6.1±0.06
	500	7.0±0.03	8.9±0.17	9.9±0.11	9.1±0.06
Crotepoxide	10	-	-	-	-
	50	-	-	-	-
	100	-	6.1±0.03	4.2±0.03	-
	500	-	8.6±0.36	7.0±0.11	3.8±0.06
Chloramphenicol	10	14.2±0.36	10.9±0.06	15.0±0.03	15.0±0.50
	50	27.0±0.17	15.2±0.25	20.0±0.03	25.7±0.11
	100	31.9±0.01	24.0±0.03	27.0±0.01	28.2±0.06
	500	34.7±0.36	27.3±0.17	30.7±0.17	31.9±0.06

SD= Standard Deviation

and *E. aerogenes* ATCC 13048, and two Gram-positive bacteria *B. cereus* ATCC 6538 and *S. aureus* ATCC. Antibacterial assays were also performed on acetone extract, *n*-hexane and ethyl acetate fractions of *K. rotunda* rhizome.

The results of antibacterial activity assay to acetone extract, *n*-hexane and ethyl acetate fraction, and isolated compounds of *K. rotunda* rhizome was indicated different inhibitory zone to all test bacteria (Table 3). All sample tests showed the lower antibacterial activity than positive control (chloramphenicol). The inhibitory zone level of bacterial growth is classified as follows: weak (< 5 mm), moderate (5–10 mm), strong (11–20 mm) and very strong (> 21 mm) [19].

Acetone extract of *K. rotunda* rhizome showed moderate activity against *E. coli* and *S. aureus* with an inhibitory zone of 8.0 and 8.1 mm at 100 μg/mL concentrations, and it exhibits high antibacterial activity (11.7 and 12.1 mm) at 500 μg/mL. While on *E. aerogenes* and *B. cereus* at 500 μg/mL showed inhibitory zone 7.9 and 10.1 mm, respectively. The *n*-hexane fraction of the *K. rotunda* rhizome showed antibacterial activity against all test bacteria. The *n*-hexane fraction of *K. rotunda* rhizome showed moderate activity at 50 μg/mL with the inhibitory zone 7.9 to 10.0 mm. The intense activity (11.1–20.2 mm) of the *n*-hexane fraction was shown at 100 μg/mL and 500 μg/mL. Both acetone extract and *n*-hexane fraction *K. rotunda* rhizome are potential as an antibacterial. The ethyl acetate fraction showed

antibacterial activity against *E. aerogenes* at a minimum concentration of 50 µg/mL with an inhibitory zone of 6.9 mm, however to the other bacteria showed weak activity.

The benzyl benzoate have lower activity than acetone extract and *n*-hexane fraction; it is suggested that the other compounds both in acetone extract and *n*-hexane fraction of *K. rotunda* rhizome have higher antibacterial activity than benzyl benzoate. Synergism is observed when the effect of combined substances is greater. Benzyl benzoate showed moderate antibacterial activity against *B. cereus* at 50–500 µg/mL with an inhibitory zone of 5.9–9.9 mm, against *E. coli* and *S. aureus* at 100–500 µg/mL with an inhibitory zone of 5.2–7.0 and 6.1–9.1 mm respectively, and to *E. aerogenes* at 500 µg/mL with an inhibitory zone of 8.9 mm.

Meanwhile, crotepoxide exhibited moderate antibacterial activity against *E. aerogenes* at 100–500 µg/mL with an inhibitory zone 6.1–8.6 mm and against *B. cereus* at 500 µg/mL with inhibitory zone 7.0 mm, while to other bacteria do not have activity. Its suggested that crotepoxide have lower activity than ethyl acetate fraction, except against *B. cereus*.

Generally, crotepoxide showed lower antibacterial activity than benzyl benzoate. This is possible because both compounds have a different structure, functional groups, and lipophilicity. The lipophilicity of compounds was affected by their ability to penetrate the cell wall of bacteria. The cell wall of bacteria has the lipid layers (lipophiles) that make these bacteria more resistant against some compounds and impermeable with limited diffusion [20]. Crotepoxide has lower lipophilicity than benzyl benzoate; therefore its ability to penetrate the bacteria cell wall was less than benzyl benzoate.

The antibacterial activities of essential oil and their components or some cyclic hydrocarbon compounds have been previously reviewed and the mechanism of action has not been studied in great detail, because most of the cyclic hydrocarbon compounds showed to have no specific cellular targets. Such as typical lipophiles, they pass through the cell wall and cytoplasmic membrane, disrupt the structure of their different layers of polysaccharides, fatty acid, and phospholipids. They can coagulate the cytoplasm and damage lipids and protein [21].

■ CONCLUSION

The benzyl benzoate and crotepoxide from *K. rotunda* rhizome were successfully isolated. The evaluation of antibacterial activity of benzyl benzoate and crotepoxide against four pathogenic bacteria confirmed that benzyl benzoate and crotepoxide have lower antibacterial activity than acetone extract and *n*-hexane fraction of *K. rotunda* rhizome. The benzyl benzoate was exhibited the highest antibacterial activity with moderate classification against *B. cereus* at a minimum concentration of 50 µg/mL and inhibitory zone 5.9 mm whereas crotepoxide showed the highest activity against *E. aerogenes* at a minimum concentration of 100 µg/mL with inhibitory zone 6.1 mm.

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