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Toxicity and Antimicrobial Activity of Zerumbon from *Zingiber zerumbet* Rhizome

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ABSTRACT. *Zingiber zerumbet* is a medicinal plant that has the potential as a source of bioactive compounds. Zerumbone is a major compound of the essential oil fraction of *Z. zerumbet*. Research on antimicrobial activity and toxicity of zerumbone is still limited. In this study, the zerumbone was isolated from *Z. zerumbet* rhizome and studied for its potential as antimicrobial agent and toxicity properties. The isolation of the zerumbone compound was carried out through extraction, fractionation and purification. The structure of zerumbone was determined by an NMR spectrophotometer (1D and 2D) and GC-MS. The toxicity assay was carried out using the Brine Shrimp Lethality Test (BSLT) method, while the antimicrobial activity assay was carried out using the microdilution method against *Escherichia coli* FNCC-0195, *Propionibacterium acnes* ATCC-11827, *Bacillus cereus* ATCC-11778, *Staphylococcus aureus* FNCC-0047, *Candida albicans*, *Malassezia furfur*, and *Aspergillus* sp. The isolation of zerumbone from the *n*-hexane fraction of *Z. zerumbet* rhizome obtained a pure compound in the form of white crystals, with melting point 65.5 °C. Toxicity assay of zerumbone was obtained LC₅₀ values of 21.29 µg/mL. The activity assays on the seven test microbes were showed potential activities with a range of MIC values of 15.62–250 µg/mL. Zerumbone has the highest activity against *Aspergillus* sp with the MIC value of 15.62 µg/mL.

Keyword: Antimicrobial, BSLT, GCMS, NMR, zerumbone,

INTRODUCTION

Traditional medicine based on biological natural resources, especially plants, has long been used in Indonesia. Therefore, it is not surprising that medicinal plants are one of the most important topics as an alternative to cure various diseases in Indonesia (Achmad et al., 2009)

Indonesia as an area with a high diversity of plant species makes it possible to find compounds that have important biological activities and pharmacological effects. One of these plants is lempuyang (*Zingiber zerumbet*.) from the Zingiberaceae family. The rhizome of *Z. zerumbet* is widely used by the community as traditional medicine, among others, to treat swelling, cough, fever, stomach pain, diarrhea, toothache, skin disease, and diuretic (Rana et al., 2012). Several studies have reported that elephant lempuyang rhizome extract has various biological activities, including antioxidant and antibacterial (Thummajitsakul et al., 2016), antimicrobial (Golam, et al., 2011), larvicidal activities against *Aedes aegypti* and *Anopheles nuneztovari* and its toxic to *Artemia salina* Leach larvae, anthelmintic (Sahu et al., 2018), immunomodulatory effect (Hardi et al., 2017), immunosuppressive effect (Ghazalee, et al., 2019), and therapeutic effect (Ahmadabadi et al., 2019).

The secondary metabolites contained in the rhizome of *Z. zerumbet* include two main groups,

namely terpenoids and flavonoids. The zerumbone compound is one of the main terpenoid compounds in *Z. zerumbet* rhizome, with the highest content ranging from 13 - 88% (Rana et al., 2012). The results showed that zerumbone has several pharmacological effects including antisecretory, gastroprotective, and antioxidant activities (Sidahmed et al., 2015), repellent (Wu et al., 2017), immunomodulatory effect (Keong et al., 2010), induce of apoptosis (Mukherjee et al., 2016), antibacterial and antiproliferative (Padalia et al., 2018), and anticancer (Abdul et al., 2008 and Koga et al., 2016). Murini et al. (2018) reported that the zerumbone which isolated from the methanol extract of *Z. zerumbet* rhizome showed good larvicidal activity, with LC₅₀ 41.75 ± 0.05 ppm and LC₉₀ 57.66 ± 3.37 ppm. Zerumbone is the main terpenoid compound in the essential oil fraction of *Z. zerumbet* rhizome.

Zerumbone exhibits several important biological activities, but research on the toxicity and antimicrobials of the zerumbone that isolated from the rhizome of *Z. zerumbet* is still limited. This research was studies the toxicity and antimicrobial activity of zerumbone that isolated from the rhizome of *Z. zerumbet*. Toxicity test was carried out on *Artemia salina* Leach larvae using the Brine Shrimp Lethality Test (BSLT) method, while antimicrobial testing was carried out on *Escherichia coli* FNCC-0195,

Propionibacterium acnes ATCC-11827, *Bacillus cereus* ATCC-11778, *Staphylococcus aureus* FNCC-0047, *Candida albicans*, *Malassezia furfur*, and *Aspergillus* sp. using the microdilution methods. Identification of isolated compounds was carried out using gas chromatography-mass spectrometer (GCMS) and 1D and 2D nuclear magnetic resonance (NMR) spectrometer.

EXPERIMENTAL SECTION

Material and Instruments

Plant material was collected from Purwokerto of Central Java, Indonesia, and identified at Botany Laboratory of Faculty of Biology, Universitas Jenderal Soedirman. Shrimp egg *A. salina* Leach, the tested bacteria (*E. coli* FNCC-0195, *P. acnes* ATCC-11827, *B. cereus* ATCC-11778, and *S. aureus* FNCC-0047), were collected from Nanobio Laboratory, Jakarta, and the tested fungi (*C. albicans*, *M. furfur* and *Aspergillus* sp), were collected from Microbiology Laboratory Faculty of Medicine, Universitas Jenderal Soedirman.

The instruments that used in this research were rotary evaporator (IKA - Digital RV8 - RV10), UV lamps 254 nm and 365 nm (CAMAG), microplate spectrophotometer for antimicrobial analysis, melting point apparatus (Melting point SMP1-Stuart Science), GCMS (GC17A MSQP 5000 Shimadzu) and NMR spectrometer (Agilent DD2: 500 (¹H) MHz and 125 (¹³C) MHz) for structure elucidation of isolated compound.

Sample Preparation

The rhizome of *Z. zerumbet* L (10 kg) was washed, cut into small pieces, and then dried at a low temperature (30-40 °C), furthermore grinded until it became powder.

Extraction and Isolation of Compounds

Z. zerumbet dried rhizome powder (1.2 kg) was macerated with acetone for 3x24 hours. Every 24 hours the extract was taken by filtering, the filtered residue was macerated again for three repetitions. The acetone extract was concentrated using a rotary evaporator. The concentrated acetone extract was fractionated by liquid-liquid partition with *n*-hexane: methanol (1:1) using a separating funnel. The dissolved extract of *n*-hexane (*n*-hexane fraction) was accommodated. The fraction of *n*-hexane was then concentrated with a rotary evaporator. Separation of *n*-hexane fraction was carried out by vacuum liquid chromatography (VLC) using silica gel column and *n*-hexane and chloroform as eluent with increased polarity (10:0; 9:1; 8:2; 7:3; 6:4; 5:5; 3:7 and 0:10). All of the VLC fractions were collected in separate bottles, and then each was concentrated with a rotary evaporator. Furthermore, each fraction was analyzed by thin layer chromatography (TLC), the fractions that showing identical spots were combined. The fraction which has one main spot and the largest weight was further purified by gravity column chromatography using *n*-hexane: chloroform (8:2) eluent. The pure

compound was identified using GCMS (Column: Rtx 5, T_{column} 70 °C, T_{injection} 300 °C, carrier gas: helium, pressure: 13.7 kPa, column flow: 0.5 mL/min, total flow : 28 mL/min) and NMR spectrometer.

Toxicity Assay

Shrimp eggs of *A. salina* Leach were hatched in a vessel containing about 1 L of seawater which was divided into two parts, the bright part which was equipped with an incandescent lamp and the dark part which was covered with black paper or carbon paper. The vessel was equipped with an aerator as a source of oxygen. One drop of yeast solution (3 mg/mL seawater) was added as a nutrient, during hatching. After 24 hours of immersion, the eggs will hatch into larvae moving towards the light. The larvae used for the BSLT test were larvae aged 48 hours after hatching (Meyer et al., 1982).

Toxicity tests were carried out on extracts, fractions and isolated compounds. Each test sample of 10 mg was dissolved in 1 mL of DMSO and then seawater was added to a volume of 10 mL (1,000 µg/mL). The stock solution was diluted and put in a test tube (vial), to obtain a final concentration of 100; 50; 25; 10; 5; and 1 µg/mL. The seawater control solution was prepared by adding 1 mL of 20% DMSO without the addition of the test sample.

Seawater containing 10 larvae of *A. salina* Leach were put in test and control tubes. The test solution and control solution were added to the tube with a certain volume then the volume of seawater was adjusted to 5 mL. The sample test and control tubes were incubated open for 24 hours. The number of surviving larvae was counted and the percentage of larvae mortality was determined to calculate the LC₅₀ value. Data analysis was carried out using the SPSS Statistics 20.0 program with probit regression analysis, with a 95% confidence interval. The program shows an estimation curve between the log concentration and the probit values, so that the LC₅₀ can be determined.

Antimicrobial Activity Assay (Diatuti et al., 2014)

The antimicrobial activity test was carried out in vitro using the liquid dilution method. The sample stock solution was prepared so that the concentration was 1000 µg/mL. The concentrations of the test samples used were 500, 250, 125, 62.5, 31.25, 15.62, 7.81 µg/mL. The sample was dissolved in 10% DMSO in distilled water.

Preparation of microbial culture

Microbes were cultured for 24 hours for bacteria, and 48 hours for fungi at 37 °C under aerobic conditions on an agar medium (Muller Hinton Agar (MHA) for bacteria and Potato Dextro Agar (PDA) for fungi). The microbes were then suspended in a 0.9% (w/v) NaCl solution and the density was equalized.

Microdilution assays

A total of 200 µL of liquid media (Muller Hinton Broth (MHB) for bacteria and Potato Dextro Broth (PDB) for fungi) was poured into each well of the microplate (96 wells). 200 µL of the test solution was

added to the first well. The solution concentration series was carried out by transferring 200 μ L of solution from the first well to the second well. From the second well another 200 μ L was taken and put into the third well. The same thing was done until to the eighth well. The amount of solution in each well was 200 μ L. Then, 10 μ L of microbial suspension was added to each well. The microplate was then incubated at 37 $^{\circ}$ C for 24 hours for bacteria and 48 hours for fungi. Microbial growth was determined using a microplate spectrometer at 600 nm. The minimum inhibitory concentration (MIC) is the lowest concentration that can inhibit microbial growth. Amoxicillin and ketoconazole were the antibiotics used as positive controls for bacteria and fungi respectively.

RESULTS AND DISCUSSION

Extraction and Isolation

Maceration is the most widely used simple extraction method. Maceration was carried out by soaking the *Z. zerumbet* rhizome powder, as well as acetone solvent into an inert container that was tightly closed at room temperature. Acetone as a solvent with medium polarity was used in the maceration process to extract nonpolar and semipolar compounds and to reduce the presence of compounds with high polarity being extracted.

The acetone extract of the *Z. zerumbet* rhizome obtained was 106.6 g (8.88% yield) as brown pasta. The fractionation of acetone extract by liquid-liquid partition with *n*-hexane: methanol (1:1) gave was 22.08 g (1.84%) of *n*-hexane fractions. Separation of *n*-hexane fraction using VLC methods get five main fractions, namely the H1 (0.34 g); H2(1.25 g); H3 (4.67 g); H4 (2.98 g) and H5 (3.27 g) fractions. Fraction H3 which have one main spot and the largest weight (4.67 g) was further purified by gravity column chromatography using *n*-hexane: chloroform (8:2) eluent, obtained seven fractions, fractions with the same profile based on TLC analysis were combined, so that three main isolates were obtained, namely H3.1 (1.76 g); H3.2 (0.27 g) and H3.3 (1.54 g). Based on TLC analysis, the H3.1 isolate was a pure compound, in the form of white crystals.

Identification of Isolated Compound.

Melting point analysis

The melting point of isolated compound was 65.5 $^{\circ}$ C. This was almost the same as the melting point of

zerumbone based on the literature, which is 65.3 $^{\circ}$ C (Girisa et al., 2019).

Gas chromatography-mass spectrometer

Gas chromatography-mass spectrometer analysis aims to determine the purity and identify it based on the molecular weight of the substance within a test sample. The GC chromatogram showed one main compound at a retention time of 28.146 minutes with a percentage area of 96.78% which indicated that the isolated compound was quite pure. The MS spectrum showed that the isolated compound had a molecular mass of m/z 218 [M+]. This indicates that the isolated compound has the same molecular mass as zerumbone ($C_{15}H_{22}O$), which is m/z 218.

Nuclear magnetic resonance (NMR) spectrometer

Analysis of zerumbone with NMR spectrometer were including analysis of 1H -NMR, ^{13}C -NMR, HSQC (Heteronuclear Single Quantum Coherence), and HMBC (Heteronuclear Multiple Bond Correlation).

The interpretation of the NMR spectra data indicate that the isolated compound was zerumbone. It has the molecular formula $C_{15}H_{22}O$, which includes four methyl groups ($-CH_3$), three methylene groups ($-CH_2-$), four sp^2 methine groups ($=CH$), one quaternary carbon sp^3 , two quaternary carbons sp^2 , and one carbonyl group. These data were compared with the previous data on NMR spectra of zerumbone (Noor & Sirat, 2016), but it has not reported the data of HSQC and HMBC. The chemical structure of zerumbone can be seen in Figure 1.

Spectral data of 1H and ^{13}C NMR of zerumbone can be seen in Table 1. Spectral data of 1H NMR showed the presence of 22 proton signals which included four methyl ($-CH_3$) signals, at δ 1.78 (s, H-12), 1.53 (s, H-13), 1.06 (s, H-14), and 1.19 (s, H-15) ppm, three signals methylene ($-CH_2-$) at δ 1.89 - 2.34 (m, H-4), 2.21 - 2.36 (m, H-5), and 2.24 - 2.43 (m, H-8)) ppm, four vinylic ($-C=C-$) proton signals at δ 6.02 (d, 11.9 Hz, H-3), 5.25 (dd, 5.5; 10.2 Hz, H-7), 5.87 (d, 16.4 Hz, H-10), and 5.98 (d, 16.4 Hz, H-11) ppm. The ^{13}C NMR spectrum data showed the presence of 15 carbon signals, namely four methyl carbons at δ of 11.91 (C-12); 15.35 (C-13); 29.56 (C-14); and 24.53 (C-15) ppm, three carbons of methylene at δ of 29.80 (C-4); 39.58 (C-5); and 42.54 (C-8) ppm, four carbons of methine sp^2 at δ of 148.95 (C-3); 125.13 (C-7); 160.87 (C-10); and

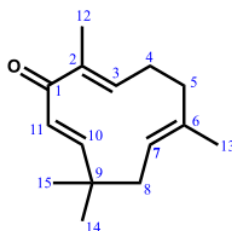


Figure 1. Zerumbone

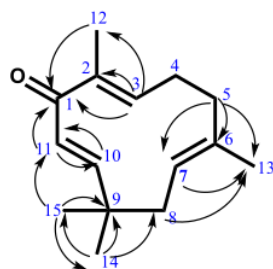


Figure 2. HMBC of zerumbone

Tabel 1. Spectra data of ^1H , ^{13}C -NMR dan HMBC of zerumbone (500 Hz, CDCl_3)

No	HSQC				HMBC ($^1\text{H} \leftrightarrow ^{13}\text{C}$)
	δH (mult. J Hz) ppm	δH^* (mult. J Hz) ppm	δC ppm	δC^* ppm	
1	-	-	204.48	204.4	-
2	-	-	138.09	137.9	6 -
3	6.02 (1H, d, 11.9)	6.02 (1H, d)	148.95	148.9	C-1, C-2 , C-12
4	1.89 – 2.34 (2H, m)	2.28 (2H, m)	29.8	29.4	
5	2.21 – 2.36 (2H, m)	2.59 (2H, m)	39.58	39.4	C-6 , C-7 , C-13
6	-	-	136.41	136.3	-
7	5.25 (1H, dd, 4.5; 14.2)	5.29 (1H, m)	125.13	124.9	C-5 , C-13 ,
8	2.24 – 2.33 (2H, m)	2.26 – 2.36 (2H, m)	42.54	42.3	C-6 , C-7 , C-13
9	-	-	37.99	37.8	-
10	5.87 (1H, d, 16.4)	5.87 (1H, d, 16.4)	160.87	160.8	C-1 , C-11
11	5.98 (1H, d, 16.4)	5.91 (1H, d, 16.4)	127.30	127.1	C-1 , C-2 , C-10
12	1.78 (3H, s)	1.82 (3H, s)	11.91	11.8	C-1 , C-2 , C-3
13	1.53 (3H, s)	1.56 (3H, s)	15.35	15.2	C-5 , C-6 , C-7
14	1.06 (3H, s)	1.09 (3H, s)	29.56	29.4	C-8 , C-9 , C-10 , C-15
15	1.19 (3H, s)	1.23 (3H, s)	24.53	24.2	C-8 , C-9 , C-10 , C-14

*(Noor & Sirat, 2016)

127.30 (C-11) ppm, three quaternary carbons at 6 138.09 (C-2); 136.41 (C-6); and 37.99 (C-9) ppm, and one carbonyl signal at 204.48 (C-1) ppm. The correlations between proton and carbons atom there were separated by two or three, based on HMBC spectrum of zerumbone was shown in **Figure 2**.

Brine Shrimp Lethality Test

Toxicity assays aimed to detect the level of toxicity of a substance/material that will be used a drug (Asaduzzaman et al., 2015). Toxicity test was carried out using the BSLT method or the shrimp larvae mortality test method. The shrimp larvae were used because it was most sensitive to the environment as their cell walls were still soft (Hamidi et al., 2014).

Artemia salina Leach was used as a target organism to detect the biological activity of bioactive compounds in *Z. zerumbet* rhizome extract. These method was closely correlated with 9KB (human nasopharyngeal carcinoma) cytotoxicity. The LC_{50} values for BSLT methods are generally about tenfold the ED_{50} values for cytotoxicities (Meyer et al., 1982). The LC_{50} values in these assays also have a significant correlation with antimicrobial activity, pesticidal effects, toxicity of the environment, and others (Asaduzzaman et al., 2015).

The LC_{50} value was obtained from the calculation of the concentration **10** against the probit value. The LC_{50} values of the **acetone extract**, the **n-hexane fraction**, and zerumbone are presented in **Table 2**.

Table 2. The LC₅₀ value of acetone extract, *n*-hexane, and zerumbone fractions from the rhizome of *Z. zerumbet* (L.)

No.	Sampel	LC ₅₀ (µg/mL)
1.	Acetone extract	7.90
2.	<i>n</i> -Hexane fraction	23.90
3.	Zerumbone	21.29

Table 3. Antimicrobial activity of acetone extract, *n*-hexane fraction, and zerumbone

Sample	MIC (µg/mL)						
	<i>E.co</i>	<i>P.ac</i>	<i>B.ce</i>	<i>S.au</i>	<i>C.alb</i>	<i>M.fur</i>	<i>Asp.sp</i>
Acetone extract	125	125	125	250	15.62	125	62.5
<i>n</i> -Hexana fraction	125	125	125	125	62.50	62.5	62.5
Zerumbone	31.25	125	125	125	62.50	31.25	15.62
Amoxicilin	31.25	31.25	15.6	3.91	-	-	-
Ketoconazole	-	-	-	-	7.81	15.62	15.62

**E.co*= *E. coli* FNCC-0195; *P.ac*=*P. acnes* ATCC-11827; *B.ce*=*B. cereus* ATCC-1177; *S.au*= *S. aureus* FNCC-0047; *C.alb*=*C.albicans*; *M.fur*=*M.furfur*; *Asp.sp*= *Aspergillus* sp;
 - = not tested

The toxicities of acetone extract, *n*-hexane fractions and zerumbone based on the Table 2 showed the LC₅₀ values were below of 1000 µg/mL. The sample is said to be toxic if it has an LC₅₀ value of less than 1000 µg/mL. The smaller the value of LC₅₀ indicates that the sample was more toxic (Meyer et al., 1982). According to Clarkson criteria for the assessment of sample toxicity are classified as follows: samples with LC₅₀ above 1000 µg/mL was non-toxic, LC₅₀ of 500-1000 µg/mL was low toxic, samples with LC₅₀ of 100-500 µg/mL was moderately toxic, and LC₅₀ of 0-100 µg/mL was very toxic. (Hamidi et al., 2014). Based on these studies, were indicates that the acetone extract, *n*-hexane fraction and zerumbone were very toxic to larvae of *A. salina* Leach, because their have LC₅₀ values of below 100 µg/mL. This indicates that they have potential pharmacological activities.

Antimicrobial Activity

Antimicrobial activity against four bacteria and three fungus of acetone extract, *n*-hexane fraction and zerumbon, was carried out using the microdilution method. The MIC values of all samples are presented in Table 3.

The data on Table 3 showed that the tested samples have activity against the seven test microorganisms, with varying levels of activity, with a range of MIC values of 15.62-250 µg/mL. In general, the antimicrobial activity of acetone extract, ethyl acetate and zerumbon fractions were showed lower activity levels than standard antibiotics (chloramphenicol and ketoconazole). Except for the activity of zerumbone against *E. coli* FNCC-0195 and *Aspergillus* sp. which showed the same activities as standard antibiotics with an MIC value of 31.25 and 15.62 µg/mL respectively.

The antibacterial activity of extracts, fractions, and zerumbon showed weak activity against *P. acnes* ATCC-11827, *B. cereus* ATCC-1177, and *S. aureus* FNCC-0047, with MIC values of 125-250 µg/mL. The assays against *C. albicans* and *Aspergillus* sp. also

showed activity in all tested samples, with MIC values of acetone extract, *n*-hexane fraction, and zerumbone, respectively, showed MIC values of 15.62, 62.50 and 62.50 µg/mL. Based on these studies was showed that acetone extract, *n*-hexane fraction dan zerumbone were more potential activities against fungi than bacteria. Wherein MIC values for tested fungi were in range of 15.62-125 µg/mL, while the MIC values for tested bacteria were in the range of 31.25-250 µg/mL.

CONCLUSIONS

The isolation of bioactive compounds from the *n*-hexane fraction of rhizome of *Z. zerumbet* was obtained pure compounds of zerumbone in the form of white crystals (m.p 65.5 °C). Toxicity assays of zerumbone by BSLT method, showed that zerumbone was toxic with LC₅₀ values of 21.29 µg/mL. The antimicrobial activity tests against *E. coli* FNCC-0195, *P. acnes* ATCC-11827, *B. cereus* ATCC-1177, and *S. aureus* FNCC-0047, *C. albicans*, *M. furfur*, and *Aspergillus* sp. showed that zerumbone has an antimicrobial activities, with a range of MIC values of 15.62-250 µg/mL. Zerumbon showed the highest activity against *Aspergillus* sp. with MIC value of 15.62 µg/mL.

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