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Antibacterial Activity of Zerumbone from Extract of *Zingiber zerumbet* (L.) Roscoe ex Smith Rhizomes

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Abstract

The rhizome of *Zingiber zerumbet*, known as Lempuyang Gajah, is commonly used in the community for medicinal purposes due to its diverse biological activities, particularly its antibacterial compounds. Among these compounds, zerumbone is one of the major secondary metabolites found in *Z. zerumbet* rhizomes. This research focused on the isolation of zerumbone and the exploration of its antibacterial properties. The isolation process involved the use of the maceration method with acetone as the solvent, while various chromatographic techniques such as Liquid Vacuum Chromatography, Column Chromatography, and Thin Layer Chromatography were employed for fractionation and purification of zerumbone. The identification of zerumbone was accomplished through GC-MS data analysis. The antibacterial activity of zerumbone was tested against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa* using the agar diffusion method. This compound exhibited moderate antibacterial activity against *P. aeruginosa* with an inhibition zone of 9.42 mm. The rhizome of *Z. zerumbet* can serve as an alternative source of zerumbone for further studies in the field of medicine.

Keywords: Antibacterial activity; Isolation; Zerumbone; *Zingiber zerumbet*

1. INTRODUCTION

Indonesia is a country that has a wealth of biodiversity. This diversity is an unlimited source of organic compounds. It is widely known that plants in the *Zingiberaceae* family mostly contain chemical compounds that can be used as medicinal plants in traditional medicine (Chavan et al., 2018; Haque et al., 2019). The Zingiberaceae family contains the genus Zingiber which has more than one type of species. One of the species of the genus *Zingiber* is *Zingiber zerumbet*, known as lempuyang gajah. *Z. zerumbet* rhizome has been widely used in traditional medicine in inflammation, diarrhea, stomach cramps, headache, toothache, asthma, carbuncles, cough, mouth ulcers, ear inflammation, gastritis, arthritis, fever, flatulence, allergies, poisoning, antioxidant and bacterial infections (Koga et al., 2016; Jantan et al., 2019; Akhtar et al., 2019; Ahmadabadi et al., 2019; Ramzan et al., 2022; Assiry et al., 2023).

Zerumbone is a major compound contained in several plants of the *Zingiberaceae* family, including *Z. spectabile*, *Z. roseum*, *Z. aromaticum*, *Z. zerumbet*, *Z. americans*, etc. According to some studies, zerumbone is mostly found in *Z. zerumbet* plants compared to other

Zingiber species. The zerumbone content in essential oil of *Z. zerumbet* is 90.62% (Mulyani, 2010), while in *Z. spectabile* it is 30% (Sadhu et al., 2007), and *Z. roseum* is 6.5% (Al-Amin et al., 2019). Researcher successfully identified the major constituents contained in essential oil from pentane extract of *Z. zerumbet* rhizomes including zerumbone (48.13%), α-humulene (17.23%), humulene epoxide I (7.88%), and humulene epoxide II (5.74%) (Yu et al., 2008).

Infectious diseases are still a health problem in both developing and developed countries. Patients with infectious diseases caused by bacteria are generally given therapy in the form of antibiotics. Inaccurate use of antibiotics can cause resistance in bacteria. This shows that a new antibacterial compound is needed that can overcome the resistance problem. Biological antibacterial agents can be obtained from fungi, bacteria, bacteriocins and plants (Prastiyanto et al., 2020). Zerumbone is one of the major compounds found in *Z. zerumbet* which is a terpenoid compound with a cyclic structure containing one oxygen atom, which gives it biological activity properties such as antibacterial, antifungal, and anti-inflammatory. In previous studies, zerumbone has been successfully isolated from the essential oil of *Z. zerumbet* rhizomes and is known to have better antibacterial activity when compared to its essential oil against *S. aureus* bacteria (MTCC 96) with an inhibition zone of 13 ± 2 mm (Padalia et al., 2018). Zerumbone from the essential oil of *Z. zerumbet* rhizomes also has potential as an antibacterial against *S. mutans* (Silva et al., 2018). Isolation of zerumbone is mostly obtained from essential oils which require methods that are not simple, so as another alternative, zerumbone isolation is carried out from *Z. zerumbet* extract. Isolation of zerumbone from *Z. zerumbet* rhizome extract is still limited and has not been widely reported. Therefore, it is necessary to isolate zerumbone from acetone extract of *Z. zerumbet* rhizome and test its antibacterial activity against gram-positive and gram-negative bacteria. Furthermore, *Z. zerumbet* can be one of the plant sources for obtaining zerumbone and can be used as a source of potential medicinal compounds.

2. MATERIAL AND METHODS

2.1. Materials

The materials *Z. zerumbet* rhizome from Pasar Gedhe, Surakarta. Technical solvents such as acetone, ethyl acetate, n-hexane. Silica gel 60 (0.040-0.063 mm, Merck, Germany) and TLC 60 PF254 (0.25 mm, Merck, Germany) were utilized for chromatography. Cerium (IV) (Merck, Germany) and H_2SO_4 (Merck, Germany) were used as spotting reagents. Dimethyl Sulfoxide (DMSO) (Merck, Germany), NaCl 0.9% (Merck, Germany), blank disks (6 mm), amoxicillin, and imipenem antibiotic disks (6 mm) were utilized in the antibacterial activity test. Four bacterial strains were obtained from the microbiology laboratory of the Faculty of Medicine, UNS, namely *Staphylococcus aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Escherichia coli* ATCC 11229, and *Pseudomonas aeruginosa* ATCC 27853. The research utilized a GCMS-QP2010S Shimadzu instrument, vacuum liquid chromatography (VLC), column chromatography, a rotary evaporator (RE-2010), and a UV254 lamp.

2.2. Isolation and identification of Zerumbone

The isolation of zerumbone from *Z. zerumbet* rhizomes, as described in research (Al-Amin et al., 2019), was conducted with slight modifications. A total of 1.75 kg of dried powdered rhizomes was extracted with acetone at room temperature, utilizing 4 L of solvent for 24 hours, with this process repeated three times. After filtering the extracting solvent, the filtrate was concentrated under reduced pressure at 40 °C using a rotary evaporator, yielding 125.73 g of crude acetone extract, corresponding to an 7.18% yield based on the dried powder. A 15 g portion of the acetone extract was subjected to fractionation through vacuum liquid chromatography (VLC) with a solvent mixture of n-hexane and ethyl acetate in various ratios (9.5:0.5; 9:1; 8:2; 7:3; 6:4; and 5:5), which yielded 17 fractions. TLC analysis guided the selection of fractions for purification. TLC plates were visualized under a UV lamp (λ254) and then treated with the spotting reagent $Ce(SO_4)_2$.

Based on the TLC analysis, 3.23 g of fraction E (FE) was purified using a mixture of n-hexane and ethyl acetate in a 9.7:0.3 ratio (250 mL), yielding 23 fractions. Fractions FE10-23 (58 mg) displayed a single spot as a white crystal. These fractions were then analyzed for structural identification using GC-MS spectroscopy.

2.3. Determination of antibacterial activity of Zerumbone

The determination of antibacterial activity followed the protocol outlined in reference (Clinical and Laboratory Standards Institute, 2018) with slight modifications. Antibacterial activity was assessed using the disc paper diffusion method. Specifically, paper discs (6 mm) were impregnated with a 20 µL mixture of pure isolate and negative control (DMSO) with varying concentrations of 16, 32, 64, 128, 256, 512 and 1024 (µg/mL). Bacterial suspension was prepared by diluting one inoculating loop of bacterial culture in 4 mL of sterile NaCl (0.9%), adjusting turbidity to the standard 0.5 McFarland standard. A single inoculating loop of bacteria was evenly spread in a zigzag pattern on Mueller-Hinton Agar (MHA) plates using aseptic techniques. Samples and reference antibiotics (Amoxicillin 20 µg/disc and Imipenem 20 µg/disc) along with a control (20 µL DMSO/disc), were evenly placed on the MHA plates and incubated for 24 hours at 35±2 °C. The inhibition of bacterial growth was determined by measuring the diameter of the inhibition zone.

3. RESULTS AND DISCUSSION

3.1. Isolation of Zerumbone

Maceration from *Z. zerumbet* rhizome powder produced a concentrated extract as a thick blackish brown extract. The *Z. zerumbet* rhizome extract (15 g) fractionation using VLC obtained 17 fractions (F_A-F_Q). Fraction E (F_E) forms white slightly light brown crystal-like solids which are thought to contain impure F_E compounds, so further purification is needed using column chromatography. The TLC profile of VLC results is shown in Figure 1.

Fraction F_E (3.23 g) from VLC was tested using TLC (Figure 2) to determine its separation pattern. The eluents used for the elution process with TLC were n-hexane: ethyl acetate with a ratio of (9: 1); (9.9: 0.1); (9.8: 0.2); (9.7: 0.3); (9.6: 0.4); and (9.5: 0.5).

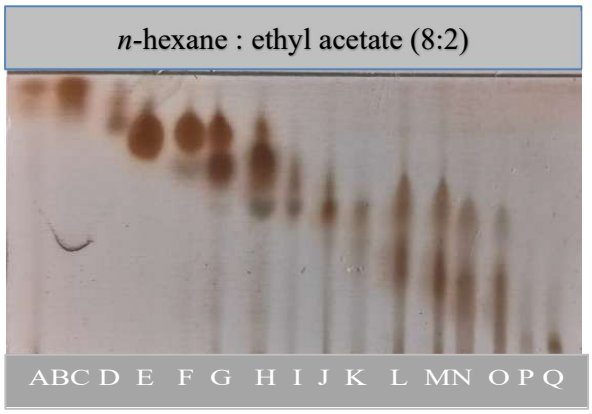

Figure 1. TLC profile of 17 fractions with n-hexane: ethyl acetate (8: 2) eluent.

Based on the TLC profile obtained in Figure 2, it can be seen that there is one elongated spot in each concentration variation. However, in this study, solvent variation was used in the ratio of 9.7: 0.3 because it was seen that the comparison produced spot stains that were lower than the other comparisons. This is done to maximize the process of separating compounds when column chromatography is performed. The results of column chromatography obtained 23 fractions which were then tested for purity using TLC. Fraction E_{1-6} is a non-polar compound because it elutes first. These compounds may be oils, fats, triterpenes, and other non-polar compounds (Ritna et al., 2016). Based on the TLC analysis, fraction E_{10-23} showed one spot on all three eluent variations and was predicted to be the target zerumbone which was further identified by GCMS. The yield of zerumbone that was successfully isolated was 1.53 g of the column chromatography results. The schematic diagram illustrating the isolation and purification process of zerumbone from the acetone extract are presented in Figure 3.

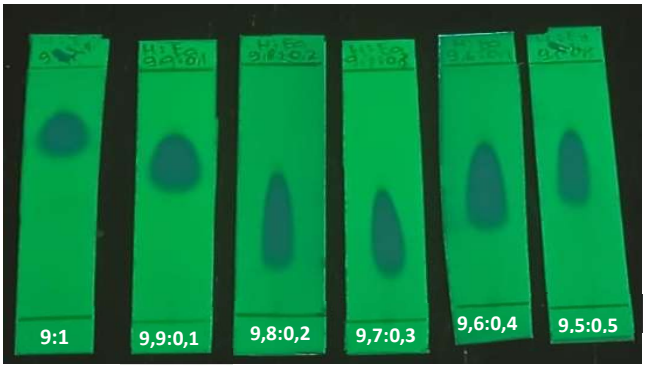

Figure 2. TLC profile of F_E with variation of n-hexane: ethyl acetate eluent ratio

The structure of fraction E_{10-23} was identified using GC-MS analysis. The GC chromatogram of isolate E_{10-23} displayed a single peak (Figure 4), indicating the presence of a single major compound. Based on the chromatogram data, it showed that the E_{10-23} isolate is pure with a retention time of 30.789 minutes and an abundance of 99.76%. The results of mass

spectra analysis of compounds detected by GC-MS contained in isolate E_{10-23} were compared with the mass spectra of standard compounds from WILEY 229.LIB.

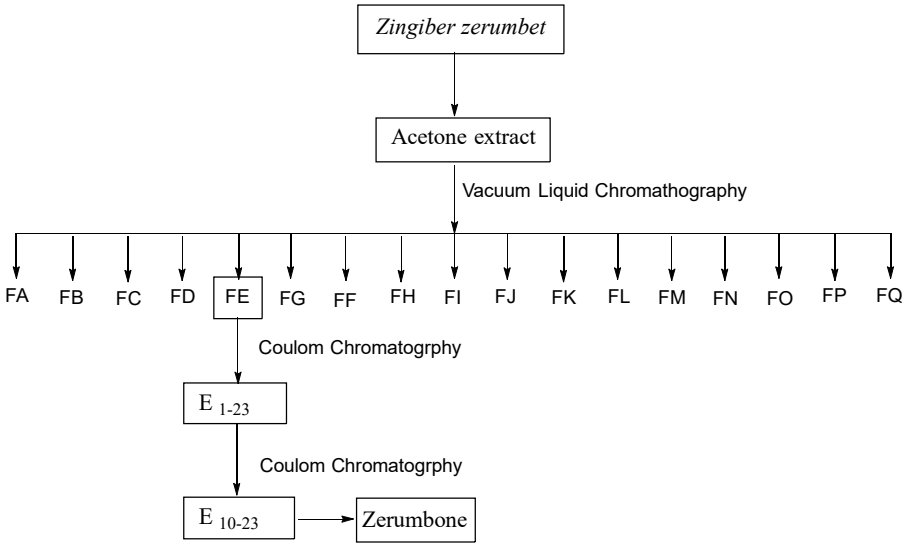

Figure 3. The schematic diagram illustrating the isolation and purification of Zerumbone from the acetone extract.

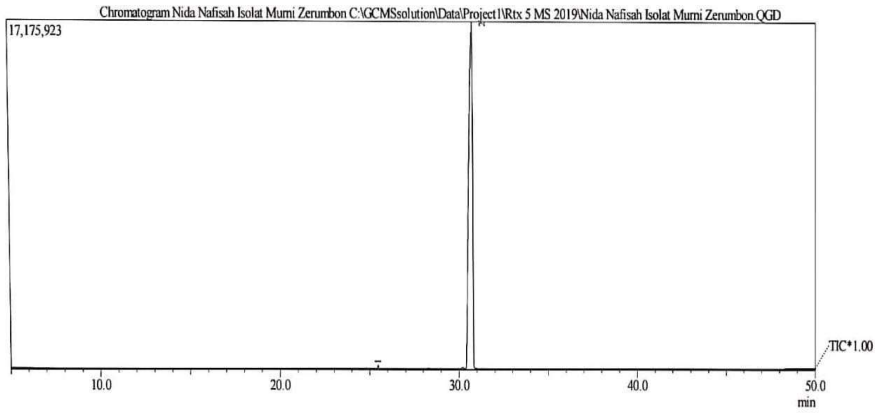

Figure 4. The GC chromatogram of compound isolate E_{10-23}.

The mass spectra of isolate E_{10-23} are shown in Figure 5a, while the mass spectra of standard compounds from the library are shown in Figure 5b. Seen in Figure 5 (a, b), the fragmentation pattern of the mass spectra of isolate E_{10-23} is almost similar to the fragmentation pattern in the mass spectra of zerumbone ($C_{15}H_{22}O$) with Similarity index (SI) 89%. Therefore, it is possible that isolate E_{10-23} is zerumbone. GC-MS data is in accordance with research conducted by Diastuti et al. (2022), which states that the GC-MS data of zerumbone obtained has a retention time of 28.146 minutes, an abundance of 96.78%, and a molecular mass of M+ 218 m/z. The mass spectrum of isolate E_{10-23} gives a peak of molecular ion M+218 which is the molecular

weight of $C_{15}H_{22}O$. Isolate $E_{10\text{-}23}$ has a base peak mass spectrum of 107 m/z. The fragmentation of compound isolate $E_{10\text{-}23}$ is shown in Figure 6.

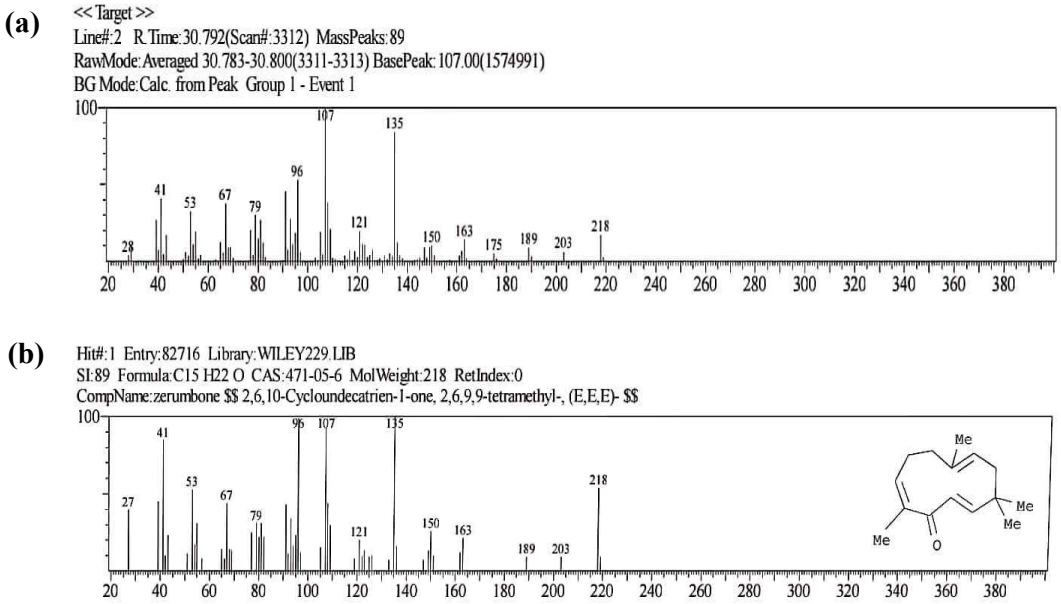

Figure 5. (a). Mass spectra of compound isolate $E_{10\text{-}23}$, (b). Mass spectra of zerumbone.

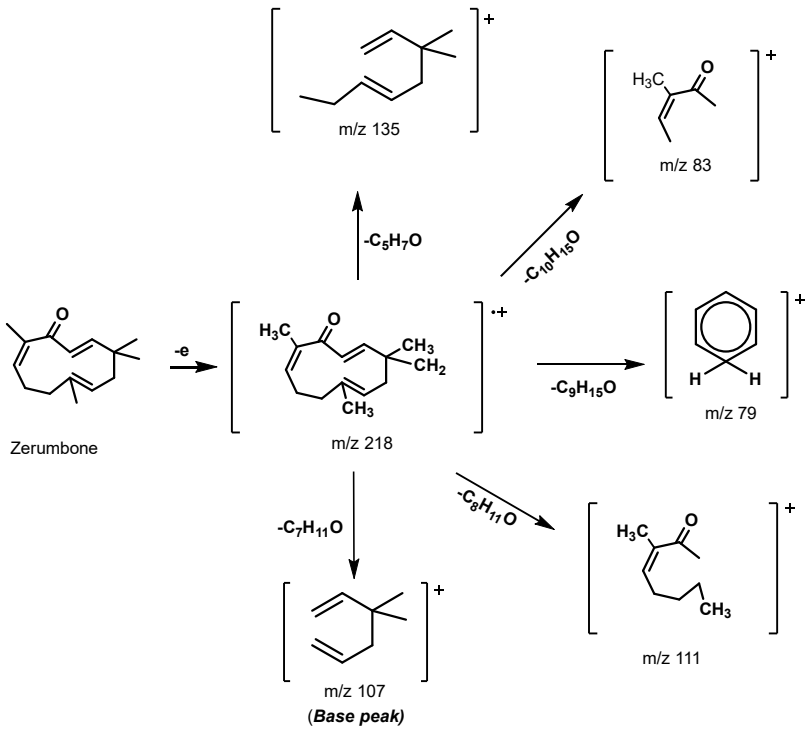

Figure 6. Zerumbone fragmentation pattern.

The fragmentation of Zerumbone results in the release of C_5H_7O, producing the $[C_{10}H_{15}]^+$ with an m/z value of 135. The release of $C_7H_{11}O$ generates the $[C_8H_{11}]^+$ with an m/z value of

107. Additionally, the release of $C_9H_{15}O$ results in the $[C_6H_7]^+$ with an m/z value of 79. The $[C_9H_{16}O]^+$ with an m/z value of 111 is formed from the release of $C_8H_{11}O$. Lastly, the release of $C_{10}H_{15}O$ produces the $[C_6H_{10}O]^+$ with an m/z value of 83.

3.2. Antibacterial in vitro testing

In the antibacterial activity test, the value of concentration is linearly proportional to the diameter of inhibition. This is in accordance with research conducted by researchers (Trisia et al., 2018), which states that one of the factors that can affect the activity of antimicrobial materials, namely the concentration of antimicrobial materials. The inhibition produced by antimicrobial materials will be higher if the concentration is also high. This can be proven through the measurement of the inhibition diameter of *P. aeruginosa* bacteria. The results of the antibacterial activity test and measurement of the inhibition zone diameter of zerumbone can be seen in Table 1 and Figure 7.

Table 1. Results of Zerumbone inhibition zone diameter measurement against *S. aureus, S. epidermidis, P. aeruginosa and E. coli* bacteria. *Description*: Disk diameter 6 mm, inoculum volume 20 µL. Negative control: DMSO.

Sample	Consentration (ppm)	Inhibition Zone Diameter (mm)			
		S. epidermidis	***S. aureus***	***E. coli***	***P. aeruginosa***
Zerumbone	16	0	0	0	7.92
	32	0	0	0	8.33
	64	0	0	0	8.42
	128	0	0	0	8.53
	256	0	0	0	8.78
	512	0	0	0	8.97
	1024	0	0	0	9.42
Amoxylline (+)		31.34	37.74	21.82	-
Imipenem (+)		-	-	-	26.66
DMSO (-)		0	0	0	0

Based on Table 1, zerumbone showed an inhibition zone of *P. aeruginosa* of 9.42 mm but showed no activity against *S. epidermidis*, *S. aureus* and *E. coli* bacteria. Al-Amin et al. (2019) reported that zerumbone had an inhibition zone of 11 mm against *P. aeruginosa*. Meanwhile, *S. aureus* and *E. coli* showed inhibition zones of 12 mm and 11 mm, respectively. The antibacterial test results revealed that zerumbone exhibits varying levels of antibacterial activity against different bacterial species, indicating that its effectiveness depends on the nature and characteristics of each bacterium. Each bacterial species has a unique level of tolerance to specific substances or compounds. According to Syarifah et al., (2018) stated that differences in the sensitivity of bacteria to antibacterial compounds can be influenced by the structure of the bacterial cell wall. Gram-negative bacteria only contain a small layer of peptidoglycan and do not contain teichoic acid, so the walls of Gram-negative bacteria are more susceptible to physical disturbances, such as antibiotics or other antibacterial agents. The mechanism of terpenoids as antibacterials is to react with the lipid fraction of the bacterial plasma membrane which results in changes in membrane permeability, if accumulated continuously it can result

in lysis of intracellular material due to the formation of cavities in the lipid bilayer (Kapitan et al., 2017).

The positive control inhibition diameter value is used as a comparison to determine the antibacterial activity of zerumbone. Classification of bacterial growth inhibition values according to Mahmudah & Atun (2017), grouped into four groups: weak response (diameter ≤5 mm), moderate (diameter 5-10 mm), strong (diameter 10-20 mm), and very strong (diameter ≥20 mm). Based on the inhibition value, it can be seen that zerumbone has moderate inhibition against *P. aeruginosa* bacteria.

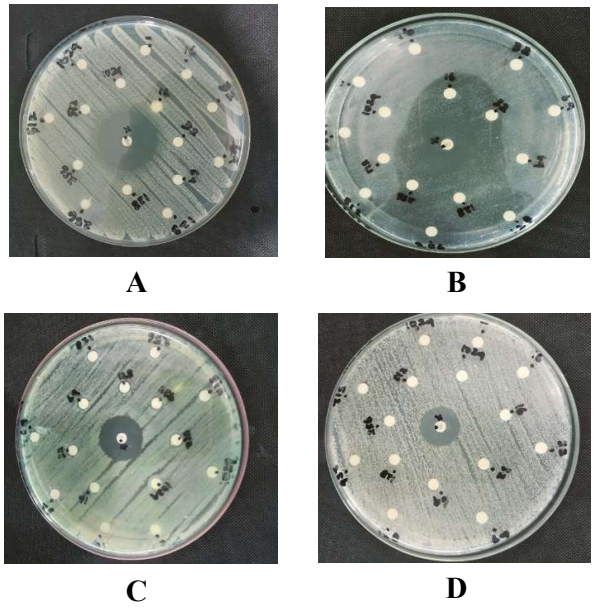

Figure 7. The antibacterial activity of zerumbone against the bacteria. *Description*: (A) *S. aureus*, (B) *S. epidermidi*s, (C) *P. aeruginosa*, and (D) *E. coli*.

4. CONCLUSIONS

Zerumbone was successfully isolated from the acetone extract of *Z. zerumbet* rhizome, resulting in a yield of 1.529 g (51% of the initial 3 g chromatography mass). This compound exhibited moderate antibacterial activity against *P. aeruginosa* forming a 9.42 mm inhibition zone, but it showed no antibacterial effect against *S. aureus*, *S. epidermidis*, or *E. coli*. The rhizome of *Z. zerumbet* could serve as an alternative source of zerumbone for further medical research.

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DECLARATION OF CONFLICT OF INTEREST

All authors declare that there is no conflict of interest in this manuscript

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