INTRODUCTION

Antibacterial substance is secondary metabolites produced primarily by microbes living in the soil. Most of these microbial form spores or cell type dormant (inactive). Allegedly, there is a connection between the metabolite production and sporulation process [1]. Thousands of microorganisms such as bacteria, fungi, and other microbes are potential as a source of secondary metabolites. It is thought to have been found in more than 50,000 compounds from microbial sources, around 23,000 of which is an active compound. Approximately 17,000 compounds from those active compounds are antibiotics. A total of 17.6 % of antibiotics produced by bacteria, 52.7 % by fungi [2-4].

For decades, microbial secondary metabolites have become the main source of new drugs. Some antibiotics in the clinic today is the result of the exploration of microorganisms. Until now, there are many new molecules of secondary metabolites derived from microorganisms that are the potential to be developed into new antibiotics [5, 6]. The important thing to do in the exploration of natural materials is to identify the microbial sources and their active compounds.

The spectroscopic analysis used to identify active compounds are sought, even some of the methods have been used for screening at the beginning of the study. Among hyphenated spectroscopic techniques, liquid chromatography-mass spectroscopy (LC-MS) and High-Performance Liquid Chromatography-Nuclear Magnetic Resonance (HPLC-NMR) are very useful for chemical compound identification [5, 7-9]. Cremin and Zeng (2002) used LC-MS to detect new metabolites of fractions as results from the partial purification of plant material [7]. This method was used by Geniloud et al. (2010) for the early dereplication process to obtain new antibiotics, namely platensimisin from Streptomyces platensis [5].

Besides LC-MS and HPLC-NMR, infrared spectroscopy (IR) is very helpful for determining the functional groups present in new secondary metabolites compounds [3-9]. Every compound produces a certain spectrum so that no two different compounds have the same spectrum. This is because the frequency of vibration is influenced by environmental bonds [1]. Some of the advantages of using IR spectroscopy is able to analyze almost any compound, many information obtained from the spectrum, relatively fast, easy, and not expensive. Additionally, IR spectroscopy is as sensitive as it can be analyze small sample sizes [12].

Identification of microbes that produce antibacterial compounds can be done by determining genetic proximity using 16S rDNA gene sequencing analysis. [13-17]. Sequence analysis of the 16S rRNA gene is a good method to determine the phylogenetic relationships between prokaryotic organisms. This technique has been used in determining the diversity of species in the genus Streptomyces [17, 18].

In this study, there is a bacteria isolate that previously showed antibacterial activity, namely J4 isolate. It was isolated from the rhizosphere of the ginger plant (Zingiber officinale) in Magelang, Indonesia. However, the J4 isolate and its antibacterial compounds have not been identified yet. J4 isolate had been detected as Gram-positive bacteria. The culture broth of J4 could inhibit the growth of tested Gram-positive and Gram-negative bacteria. Therefore, it is necessary to identify the name of bacteria J4 isolate and to characterize the active metabolite in J4 isolate.

MATERIALS AND METHODS

Identification of J4 bacteria isolates based on the 16S rRNA gene

Preparation of culture in liquid media

A total of 0.5 ml isolates culture in SNB was a subculture in 5 ml of SNB media, and then incubated for 5 d at room temperature. Subsequently, it was used for DNA isolation.

Genomic DNA extraction

A total of 1 ml culture media SNB was centrifuged, washed with TE 0.4 ml, and resuspended in SET buffer (75 mmol Na3 (Sigma,
with a mobile phase of chloroform-methanol (7:3 v/v). The detection was done with UV at 254 nm and 366 nm as well as with vanillin-sulfuric acid spray reagent. Test bacterial suspension is spread on Mueller Hinton Agar on a petri dish, and was then the TLC plate was placed on the media for 30 min. After the TLC plate was reinstated, petri dish is resoaked and incubated for 18-24 h at a temperature of 37 °C. Areas that showed an inhibition zone were measured as the distance from the start point and its Rf value is calculated.

Chemical analysis of metabolite in the active fraction

The active fraction was subjected to FTIR spectral measurement using the FTIR spectrophotometer (Shimadzu, Kyoto, Japan) in KBr disk at wavenumbers of 4000-400 cm⁻¹. The measurement was performed in controlled room temperature (20 °C). Characterization of the fraction was also conducted using UV spectroscopy (Shimadzu, Kyoto, Japan), HPLC (Shimadzu, Kyoto, Japan), TLC Scanner (Camag 4, Muttenz, Switzerland), and LC-TOF-MS.

RESULTS AND DISCUSSION

Identification of bacterial isolate

Sequence analysis of the 16S rRNA gene is a good method to determine the phylogenetic relationships between prokaryotic organisms and has been used in determining the species diversity of microorganisms [18]. Therefore, in this study, the results of 16S rRNA gene sequencing were used to identify the bacterial J4 isolate. The identification using BLAST analysis can show the similarity of gene sequences compared to gene sequences contained in the database Gene bank. The identification results are used to determine the proximity of bacterial isolates that were elected to the database of existing microorganisms.

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Prior to sequencing, PCR was first performed to duplicate DNA. PCR was conducted using primers: 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492 (5'-TACGG [A/T/C] TACCTTGTTAGACTT-3'). PCR was performed in conditions: pre-denaturation: 3 min at a temperature of 96 °C, 30 cycles of PCR [denaturation: 1 min at a temperature of 94 °C annealing: 1 min at a temperature of 53 °C extension: 5 min at 72 °C, the extension finals: 5 min at a temperature of 72 °C. According to the BLAST analysis of the 16S rRNA gene, J4 isolate has a close sequence with Burkholderia sp with the identical percentage of 99%. One of these species is Burkholderia (Pseudomonas) cepacia that produces phenylpyruvole, a broad-spectrum antimicrobial compound called pyrrolinirin [3-chloro-4-[2'-nitra-3'- chlorophenyl] Pyrrole] with the chemical formula ClO86C2N2O2 [20]. These compounds are active against filamentous fungi, yeast, and Gram-negative bacteria. It also produces antibiotics cepacin A and B that can inhibit Gram-positive and negative bacteria [21, 22]. The closeness of the 16S rRNA gene sequence between isolate J4 and Burkholderia sp was 99%, therefore it can be concluded that J4 is a species of Burkholderia sp.

Antibacterial activity of extract

Antibacterial activity test of ethyl acetate extract of Z. officinalis 20%w/v in 10% DMSO using cup plate showed the growth inhibition of S. aureus and E. coli with inhibition zone diameter of 11.8 and 21.3 cm (including the well diameter 0.6 cm), respectively. In contrast to the negative control, wells with 10% DMSO indicated no inhibition zone. To screen metabolites possessing antibacterial activity, a TLC bioautography was performed. The active zone on certain Rf value will appear if the spots contain certain levels of antibacterial compounds [23, 24]. In this study, the comparison Rf values of compounds that have active as against S. aureus and E. coli was used as well as the positive control (chloramphenicol 0.675 mg) is depicted in Fig. 1. The active zone appeared at Rf 0.76 against both S. aureus and E. coli.

TLC-bioautography of extract fractions

Based on the bioautography results, the culture fluid extract of isolate J4 showed an inhibition zone against S. aureus and E. coli with the same Rf value. The TLC bioautography results of the antibacterial activity of the extract fractions only used S. aureus as the tested bacteria. The results showed that around the hexane, chloroform-methanol (7:3 v/v), and methanol fractions, the active zone only appeared in the chloroform-methanol fraction with the same Rf value as the active spot Rf in the extract, namely 0.76. Fig. 2
demonstrated the active zone resulted from the TLC bioautography and comparison of the TLC profile between extract and chloroform-methanol fraction (called CM fraction) under UV light 254 nm and 366 nm. The TLC profile of extract showed the appearance of many spots under UV 254 nm or UV 366 nm, while the CM fraction was only detected 1 spot under 366 nm but there was no spot under 254 nm. It can be pointed out that the fractionation process was well conducted because many metabolites could be eliminated.

Fig. 1: The active zone of J4 extract against *S. aureus* or *E. coli* was at Rf 0.76; while Chloramphenicol as a positive control was at Rf 0.83

![Image](image1.png)

Fig. 2: The active zone of TLC bioautography of CM fraction (A) and the TLC chromatogram of CM fraction under UV light 254 nm (B) and 366 nm (C) as well as extract under UV light 254 nm (D) and 366 nm (E)

Chemical compound analysis

Analysis of active compounds was carried out using the CM fraction as a sample. This was because, in the TLC analysis of the CM fraction, only 1 spot appeared. Besides that, it is also due to the limited yield of the resulting CM fraction. To observe the purity of fraction CM, it was carried out HPLC analysis. The analysis was performed by HPLC using a mobile phase with the concentration series of methanol 100%, 90%, 80%, 70%, 60%, and 50%. The results of the HPLC analysis of the active fraction J4 shows that the fraction is not pure. Although there was only one peak when using 100-70% of methanol in the use of 60% and 50% methanol, it appeared that there was more than 1 peak. The separation profile in the use of methanol 60% as a mobile phase is shown in fig. 3 and table 1. This fraction also produced two major peaks at RT of 2.5 and 2.8 min with % area of 31.8% and 60.1% respectively. Besides, it is also detected two peaks with low intensity with retention times of 1.9 and 5.5 min. The two major peaks are at adjacent retention times, namely at min 3.5 and 3.8. This shows that both of them have almost the same polarity so that in the previous TLC examination, the two components were in the same spot. Based on table 1, the area percentage of these two major spots is 31.8% and 60.1% or the total of both is equal to 91.9%.

![Image](image2.png)

Fig. 3: Separation profile of CM fraction by HPLC in the use of methanol 60% as the mobile phase
Table 1: Data of HPLC chromatogram of CM fraction with mobile phase of methanol 60%

| Peak | Retention time | Area  | Height | Area % | Height % |
|------|----------------|-------|--------|--------|---------|
| 1    | 1.9            | 62090 | 3852   | 6.1    | 8.8     |
| 2    | 2.5            | 324196| 18452  | 31.0   | 42.1    |
| 3    | 2.8            | 21281 | 451    | 60.1   | 100.0   |
| 4    | 5.5            | 21265 | 451    | 2.0    | 1.0     |
| Total|                | 1020572| 43837  | 100.0  | 100.0   |

The next analysis used IR Spectroscopy. The FTIR spectrum CM fraction (fig. 4) showed OH stretching vibration frequency in the wave numbers of 3300 to 3500 cm⁻¹ in which a broad peak indicates hydrogen bonds. The aliphatic CH stretching vibrational frequencies in the region 2930-2980 cm⁻¹ and a strong absorption peak at 1640 cm⁻¹. Some peaks are also detected in wave numbers of 308.61; 347.19; 401.19; 478.35; 709.8; 763.81; 1056.99; 1273.02; 1381.03; 1635.64; 2121.7; 2931.8; 2978.09; 3302.13; 3456.44; and 3857.63 cm⁻¹.

Fig. 4: IR Spectra of CM fraction at wave numbers of 4,000-400 cm⁻¹

Furthermore, LC-TOF-MS analysis of the CM fraction was used to estimate the chemical formula of the components contained in the fraction. The LC-TOF-MS Chromatogram of the CM fraction is shown in fig. 5 and the m/z data of each chemical formulas are summarized in table 2. There were 3 peaks detected in the CM fraction, which appeared at the retention times 2.94; 9.34, and 10.34 min. The chemical formula for RT of 2.94 was C₆H₁₂N₁₂ with m/z 253. RT 9.34 shows the formula C₂₁H₂₉N₃O₅ with m/z 404 as well as C₂₁H₂₆N₂O₅ and C₁₇H₂₂N₈O₃ with m/z 387, while RT 10.34 is C₁₅H₃₅N₃O with m/z 274.

Fig. 4: LC-TOF-MS chromatogram of CM fraction, the oval circle shows the peaks of the compound contained in the CM fraction

Table 2: The molecular formula and the m/z value detected in CM fraction by LC-TOF-MS

| No | RT   | Formula          | m/z  | err (mDa) | mSigma |
|----|------|------------------|------|-----------|--------|
| 1  | 2.94 | C₆H₁₂N₁₂         | 253.1| -3.7      | 9.2    |
| 2  | 9.34 | C₂₁H₂₉N₃O₅      | 404.2| 3.6       | 3.0    |
|    |      | C₂₁H₂₆N₂O₅      | 387.2| 3.9       | 6.3    |
|    |      | C₁₇H₂₂N₈O₃      | 387.2| -1.2      | 7.7    |
| 3  | 10.34| C₁₅H₃₅N₃O       | 274.3| 4.7       | 3.8    |
Based on the IR analysis which showed the presence of OH stretching, the molecular formula of C₉H₉N₁₂ with a retention time of 2.94 min and m/z of 253 is different from the other molecular formulas, because there is no OH group. Therefore, there are 4 possible molecular formulas having OH groups produced by J4 bacteria, namely C₂₁H₂₉N₃O₅, C₂₁H₂₆N₂O₅, C₁₇H₂₂N₈O₃, or C₁₅H₃₅N₃O. These molecular formulas differ from the molecular formulas of pyrrolnitrin, cepacin A, and cepacin B, the previously discovered antimicrobial compounds produced by Burkholderia sp [20, 22].

The limitation of this study is that it has not been further tested on the molecular structure of the active substance. Therefore, in the next research, it is necessary to determine the molecular structure of the active substance.

CONCLUSION

The J4 bacteria isolate was detected as Burkholderia sp. and produce the antibacterial compound with the possible molecular formulas of C₂₁H₂₉N₃O₅, C₂₁H₂₆N₂O₅, C₁₇H₂₂N₈O₃, or C₁₅H₃₅N₃O. The real molecular structure of this formula needs to be determined in the next study.

ABBREVIATION

LC-TOF-MS (Liquid Chromatography-Time of Light-Mass Spectrometry), IR (Infra-Red), RNA (Ribonucleic Acid), BLAST (Basic Local Alignment Search Tool), HPLC (High-Performance Liquid Spectrometry), IR (Infra-Red), RNA (Ribonucleic Acid), BLAST (Basic Local Alignment Search Tool), HPLC (High-Performance Liquid Spectrometry), SNA (Starch Nitrate Agar), SNB (Starch Nitrate Broth), TLC (Thin Layer Chromatography), DMSO (Dimethylsulfoxide), FTIR (Fourier transform infrared spectroscopy).

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

The authors reported no conflict of interest. The authors are responsible for the content and writing the paper.

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