International Journal of Applied Pharmaceutics

ISSN- 0975-7058 Vol 13, Special Issue 2, 2021

Original Article

ANTIBACTERIAL SUBSTANCE PRODUCED BY A SOIL BACTERIA ISOLATED FROM RHIZOSPHERE OF *ZINGIBER OFFICINALE*

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Received: 28 Aug 2020, Revised and Accepted: 07 Oct 2020

ABSTRACT

Objective: In our previous study, we have found many isolates of bacteria from *Zingiber officinale* rhizosphere in Magelang, Central Java, Indonesia. J4, one of the isolates, has been found to contain metabolites which have antibacterial activity. The active chemical compound was unidentified. This study aims to identify the molecular formula of the active compound which has potential antibacterial activity.

Methods: Identification of selected bacteria (J4 isolate) was based on the 16S rRNA gene sequence. Extraction of J4 isolate culture broth was carried out using ethyl acetate and followed by fractionation with hexane, chloroform-methanol (7:3), and methanol. The fraction which has antibacterial activity analyzed using IR Spectroscopy and LC-TOF-MS.

Results: BLAST analysis result of the 16S rRNA sequence showed that J4 isolate is *Burkholderia* sp with 99% similarity. According to the IR spectroscopy examination of the active fraction, there were OH, CH, and carbonyl stretching. LC-TOF-MS analysis showed 5 molecular formulas with m/z of 253, 274, 387 (two formulas), and 404 in the active fraction, but there was one formula with no OH groups.

Conclusion: J4 isolate is a *Burkholderia* sp. The molecular formula for the antibacterial substance that might be produced by J4 isolate is $C_6H_{12}N_{12}$, $C_{21}H_{29}N_3O_5$, $C_{21}H_{26}N_2O_5$, $C_{17}H_{22}N_8O_3$, and/or $C_{15}H_{35}N_3O$.

Keywords: Antibacterial substance, Rhizosphere, *Burkholderia* sp, 16S rRNA

DOI: http://dx.doi.org/10.22159/ijap.2021.v13s2.12 Journal homepage: https://innovareacademics.in/journals/index.php/ijap

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 *Actinomycetales*, and 29.7 % 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 Genilloud *et al.* (2010) for the early dereplication process to obtain new antibiotics, namely platensimisin from *Streptomyces platensin* [5].

Besides LC-MS and HPLC-NMR, infrared spectroscopy (IR) is very helpful for determining the functional groups present in new secondary metabolites compounds [10]. 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 [11]. 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 officinalle*) 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 NaCl (Sigma,

Aldrich, USA), 25 mmol EDTA (Sigma, Aldrich, USA), 20 mmol Tris pH 7.5 (Sigma, Aldrich, USA). Lysozyme (Biobasic, Markham, Canada) was added with approximately 50 µl (10 mg/ml) and incubated at 37 °C for 1 h. Approximately 50 µl 10% SDS was added and incubated 65 °C for 1 h then 50 µl NaCl 5 M was added and incubated at 65 °C for 1 h. The mixture was added with 400 µl chloroform and incubated at room temperature for 30 min. The mixture is subjected to centrifugation at 13000 rpm for 10 min and the aqueous phase was transferred to a new tube. Chromosomal DNA was precipitated using 1 volume of 2-propanol. DNA is transferred to a new tube, washed with 70% ethanol, dried, and dissolved with the appropriate volume of TE buffer.

Analysis of 16S rRNA gene

Analysis of 16S rRNA gene was performed by 16S rRNA gene amplification and DNA Sequencing using primer specific for bacteria 27F: (5'-AGAGTTTGATCCTGGCTCAG-3') and R 1 4 9 2: (5'-TACGG [A/T/C] TACCTTGTTACGACTT-3') (Biobasic, Markham, Canada). PCR mixture contains 2 µl H_2O, 1 µl genomic DNA as a template (50 ng/µl), 1 µl each primer (15 pmol), and 5 µl Master Mix (Fermentas, Massachusetts, USA). PCR was run 30 cycles at 80 °C for 5 min as initial temperature, 0.5 min at 94 °C for DNA denaturation, 0.5 min at 55 °C for annealing, 1 min 72 °C for extension and 72 °C for 7 min as the final temperature. PCR results were subjected to gel electrophoresis with agarose 1% and then was blotted using ethidium bromide. DNA Sequencing was performed on PCR products in Macrogen, Korea. Sequencing results were analyzed by BLAST (NCBI) to determine the similarity of isolates with the International database of NCBI (National Center for Biotechnology Information) [19].

Preparation of metabolite extract

2 plugs of culture in media SNA (each liter contains 20 g Soluble Starch, 1 g KNO_3, 0.5 g $K_2HPO_4.3H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.5g NaCl, 0.01 g $FeSO_4.7H_2O$, 20 g bacteriological agar, and aqua dest) was subculture in 50 ml media SNB (starch nitrate broth, containing SNA media, but without agar) and incubated at room temperature (25-30 °C) for 5 d. A total of 30 ml of liquid culture was transferred into 300 ml medium SNB and incubated 5 d at room temperature, and then transferred again to 2.7 L SNB media and incubated for 14 d at room temperature. Furthermore, the culture fluid was filtered, and the filtrate was extracted with ethyl acetate followed by evaporation to obtain ethyl acetate extract.

Fractionation of metabolite extract

Fractionation of the active extract was conducted by the trituration technique. The extract was first dissolved in methanol and then combined with silica gel powder 60 for column 0040-0063 mm (Merck, Darmstadt, Germany), and then dried. Fractionation was done successively with hexane, chloroform-methanol (7:3 v/v), and methanol. Each fraction was then evaporated to dryness.

Antibacterial activity assay against *S. aureus* and *E. coli*

Preparation of test bacteria (S. aureus and E. coli)

A-1 ose bacteria were taken and added with 1 ml BHI (Bio Merieux, Marcy-l'Étoile, France) and then incubated at 37 °C for 18-24 h. After that, it was taken 100 µl and put into a tube and added with 1 ml of BHI, and then incubated at 37 °C for 3-5 h. Furthermore, the result of incubation was diluted with NaCl 0.9% to the same clarity with Mc Farland standard of 10^8 CFU/ml to obtain a bacterial suspension test.

Antibacterial activity test by cup plate method

Test bacterial suspension was spread on Media for Mueller Hinton (Merck, Darmstadt, Germany), and made wells with a diameter of 6 mm using a cork borer. Furthermore, wells are filled with 50 µl ethyl acetate extract 20% w/v in 10% DMSO and incubated for 18-24 h at a temperature of 37 °C. While chloramphenicol was used as positive control. Sterile zone formed was measured as inhibition zone diameter.

Detection of antibacterial TLC spot by bioautography assay

A total of 1.25 mg of extract was dissolved in methanol, and then spotted into TLC plate silica gel F 254 (Merck, Darmstadt, Germany) and eluted 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 onthe media for 30 min. After the TLC plate was reinstated, petri dish is resealed 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 R_f 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.

Prior to sequencing, PCR was first performed to duplicate DNA. PCR was conducted using primary i.e. 27F (specific for Bacteria) (5'-AGAGTTTGATCCTGGCTCAG-3') and R1492 (5'-TACGG [A/T/C] TACCTTGTTACGACTT-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 phenylpyrrole, a broad-spectrum antimicrobial compound called pyrrolnitrin [3-chloro-4-(2'-nitro-3'-chlorophenyl) Pyrrole] with the chemical formula C10H6Cl2N2O2 [20]. These compounds are active against filamentous fungi, yeast, and Gram-positive 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. officinalle* 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 mm), 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 patches that have activity against *S. aureus* or *E. coli* 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, so the TLC-bioautography analysis of the antibacterial activity of the extract fractions only used *S. aureus* as the tested bacteria. The results showed that among 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.

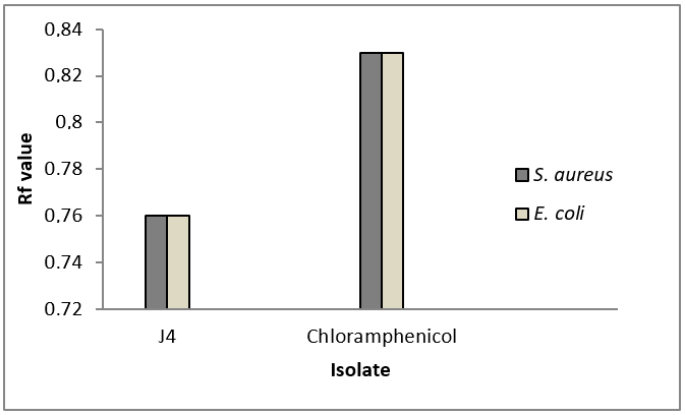

Fig. 1: The active zone of J4 extract against *S. aureus* or *E. coli* was at Rf 0.76; while Chloramphenicol as a positive control was at Rf 0.83

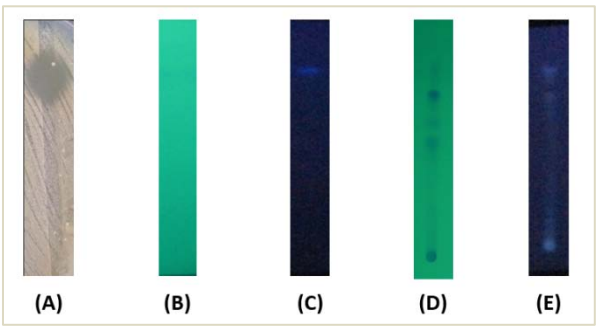

Fig. 2: The active zone of TLC bioautography of CM fraction (A) and the TLC chromatogram of CM fraction under UV light 254 nm (B) and 366 nm (C) as well as extract under UV light 254 nm (D) and 366 nm (E)

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%.

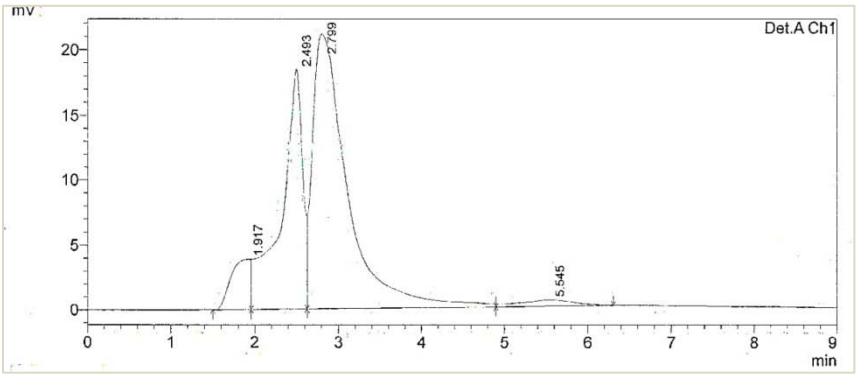

Fig. 3: Separation profile of CM fraction by HPLC in the use of methanol 60% as the mobile phase

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	31.8	42.1
3	2.8	613591	21081	60.1	48.1
4	5.5	20695	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}.

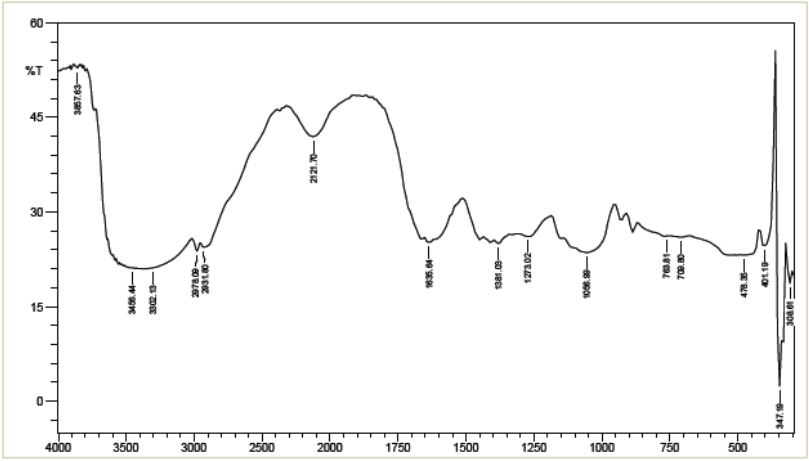

Fig. 4: IR Spectra of CM fraction at wave numbers of 4,000-400 cm^{-1}

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 $C_6H_{12}N_{12}$ with m/z 253. RT 9.34 shows the formula $C_{21}H_{29}N_3O_5$ with m/z 404 as well as $C_{21}H_{26}N_2O_5$ and $C_{17}H_{22}N_8O_3$ with m/z 387, while RT 10.34 is $C_{15}H_{35}N_3O$ with m/z 274.

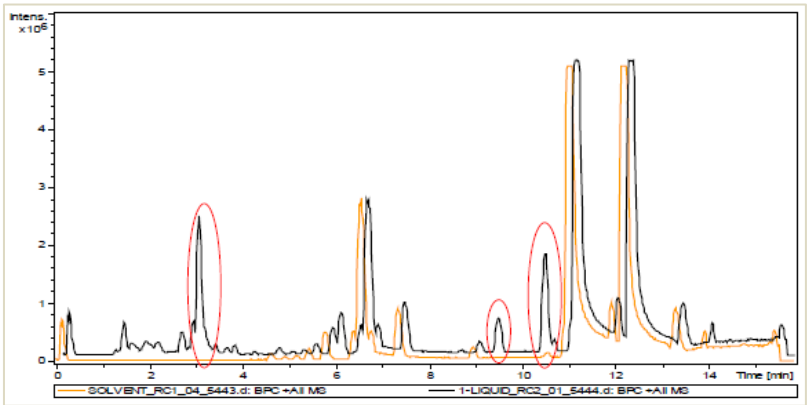

Fig. 4: LC-TOF-MS chromatogram of CM fraction, the oval circle shows the peaks of the compound contained in the CM fraction

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	$C_6H_{12}N_{12}$	253.1	-3.7	9.2
2	9.34	$C_{21}H_{29}N_3O_5$	404.2	3.6	3.0
		$C_{21}H_{26}N_2O_5$	387.2	3.9	6.3
		$C_{17}H_{22}N_8O_3$	387.2	-1.2	7.7
3	10.34	$C_{15}H_{35}N_3O$	274.3	4.7	3.8

Based on the IR analysis which showed the presence of OH stretching, the molecular formula of $C_6H_{12}N_{12}$ 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 $C_{21}H_{29}N_3O_5$, $C_{21}H_{26}N_2O_5$, $C_{17}H_{22}N_8O_3$, or $C_{15}H_{35}N_3O$. 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 $C_6H_{12}N_{12}$, $C_{21}H_{29}N_3O_5$, $C_{21}H_{26}N_2O_5$, $C_{17}H_{22}N_8O_3$, and/or $C_{15}H_{35}N_3O$. 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 Chromatography), SNA (Starch Nitrate Agar), SNB (Starch Nitrate Broth), TLC (Thin Layer Chromatography), DMSO (Dimethylsulfoxide), FTIR (Fourier-transform infrared spectroscopy).

ACKNOWLEDGEMENT

We would like to thank Universitas Ahmad Dahlan for supporting the facilities of research

FUNDING

This research was funded by Universitas Ahmad Dahlan.

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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