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Judul :

"ANTIBACTERIAL SUBSTANCE PRODUCED BY A SOIL BACTERIA ISOLATED FROM RHIZOSPHERE OF *Zingiber officinale*"

Berikut rangkuman korespondensi :

Tanggal	Korespondensi		Hal
	Author	Committee	
31 Agustus 2020		Pengiriman LoA atas pengiriman artikel (lampiran 1. Bukti email dan lampiran LoA)	2
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24 September 2020	Email pengiriman artikel hasil perbaikan (Lampiran 3. Bukti Email dan lampiran artikel)		17
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Lampiran 1. Email pemberian Loa atas pengiriman artikel dan Lampiran LoA

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Dear Participant,

Thank you for the support to The 3rd Postgraduate Seminar in Pharmaceutical Sciences. This is LoA Abstract submission in the attached file, please complete your payment registration and thanks for your participation.

You are warmly welcomed to participate in the conference: Thursday, September 3rd, 2020 on zoom platform.

Best Regards
The 3rd PSPS 2020 Committee

66. LoA PSPS_NANIK SULISTYANI.pdf
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Jakarta, August 30th 2020
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LETTER OF ACCEPTANCE

Dear Nanik Sulistyani,

The Committee Of The 3rd Post Graduate Seminar In Pharmaceutical Sciences (The 3rd Psps) "**Antibacterial Substance Produced by a Soil Bacteria Isolated from Rhizosphere of *Zingiber Officinale***" Has Been Reviewed And Accepted By The Scientific Committee **Poster Presentation**, Scheduled On September 3rd 2020 At Zoom Platform.

Congratulation on the acceptance of your abstract and we look forward to your full participation on the conference and inform you password and zoom link by an email.

Yours sincerely,

Dr. apt. Yati Sumiyati, M.Kes
Chairwoman of the Organizing Committee
Pharmacy Faculty, Universitas Pancasila
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Lampiran 2. Email tentang perlunya revisi artikel dan Lampiran Artikel

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Review article 3rd PSPS

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Kepada: naniksulistyani@gmail.com

Dear Dr. apt. Nanik Sulistyani, MSi,

Thank you for your participation in The 3rd Postgraduate Seminar in Pharmaceutical Sciences 2020.

Your article is quite interesting but it needs minor revision. Overall the manuscript is interesting and results are encouraging. After an initial review, we decided that your manuscript " ANTIBACTERIAL SUBSTANCE PRODUCED BY A SOIL BACTERIA ISOLATED FROM RHIZOSPHERE OF ZINGIBER OFFICINALE" is suitable for publication in the International Journal of Applied Pharmaceutics (Int J App Pharm), Scopus Q3 SJR 0.22, The full article, special issue will be published in January 2021. Please prepare for article publication fee: 400USD/article. The submission to IJAP will be organized by 3rd PSPS committee.

For revision of your article see the following points

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

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ABSTRACT

Objective : In our previous study, we have found many isolates of bacteria from *Zingiber officinale* rizosphere in Magelang, Central Java, Indonesia. J4, one of its isolate has been known had a metabolite which has antibacterial activity. The active chemical compound was unidentified. The aim of this study is 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 the culture broth of J4 isolate using ethyl acetate followed with fractionation. The fraction which has antibacterial activity analyzed using IR Spectroscopy and LC-TOF-MS.
Results : BLAST analysis result of 16S rRNA sequence showed that J4 isolate is *Burkholderia sp* with 99% similarity. According to its IR spectroscopy examination, there were OH, CH and carbonyl stretching. LC-TOF-MS analysis showed 5 molecular formula with m/z of 270, 274 and 404 in the active fraction, but there was one formula with no OH groups.
Conclusion : J4 isolate is a *Burkholderia sp*. The active substances molecular formulas produced by J4 isolate are C21H29N3O5, C21H26N2O5, C17H22N8O3 or C15H35N3O.

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

INTRODUCTION

Antibacterial substance are secondary metabolites produced primarily by microbes living in the soil. Most of these microbials 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 [2,3,4].

For decades, microbial secondary metabolites 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 potential to be developed into new antibiotics [5,6]. The important thing to do in the exploration of natural materials is to identify the source and to identify the active compounds present.

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 hypenated specroscopic techniques, liquid chromatography - mass spectroscopy (LC-MS) and *High Performance Liquid Chromatography-Nuclear Magnetic Resonance* (HPLC-NMR) were very usefull for chemical compound identification [5,7-9]. Cremin & 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 early dereplication process in order 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 compound. Each compound gave specific spectrum, so that there are no two different compounds which have the same spectrum. This is because the frequency of vibration is influenced by environmental bond [10]. Some advantages of using IR spectroscopy is able to analyze almost any compound, much of the information obtained from the spectra, relatively quick, easy and inexpensive. In addition, IR spectroscopy is sensitive because it can analyze the small sample size [11].

Identification of microbial producers of antibacterial active compound can be done by analyzing the diversity of microbial isolates by 16S rDNA gene sequencing analysis [12-16]. Sequence analysis of 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 [16,17].

In this study, there is a bacteria isolate which 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 a Gram-positive bacteria. The culture broth of J4 could inhibit the growth of 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 isolate based on the 16S rRNA gene

Preparation of culture in liquid media

A total of 0.5 mL isolates culture in SNB was subcultured in 5 mL of SNB media, and then incubated for 5 days 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 mM NaCl (Sigma, Aldrich, USA), 25 mM EDTA (Sigma, Aldrich, USA), 20 mM Tris pH 7.5 (Sigma, Aldrich, USA). Lysozim (Biobasic) was added with approximately 50 µL (10 mg/mL) and incubated at 37°C for 1 hour. Approximately 50 µl 10% SDS was added and incubated 65°C for 1 hour then 50 µL NaCl 5 M was added and incubated at 65°C for 1 hour. The mixture was added with 400 µL chloroform and incubated at room temperature for 30 minutes. 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 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). 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). Temperature PCR was set as follows: initial temperature is 80°C for 5 minutes at, then 30 cycles for 0.5 minute at temperature of 94°C, 0.5 minute at 55°C, 1 min 72°C and final temperature was set at 72°C for 7 minute. 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).

Preparation of metabolite extract

A total of 2 plug culture in media SNA (each litre 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 aquadest) was subcultured in 50 mL media SNB (starch nitrate broth, containing SNA media, but without agar) and incubated at room

temperature (25-30°C) for 5 days. A total of 30 mL of liquid culture was transferred into 300 mL medium SNB and incubated 5 days at room temperature, and then transferred again to 2.7 L SNB media and incubated for 14 days at room temperature. Furthermore, the culture fluid is 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 trituration technique. Extract was first dissolved in methanol and then combined with silica gel powder 60 for column 0040-0063 mm (Merck, Darmstat, Germany), and then dried. Fractionation is done successively with hexane, chloroform - methanol (7: 3 v/v), and methanol. Each fraction is 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 was taken and added with 1 mL BHI (Bio Merieux and then incubated at 37°C for 18-24 hours. 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 hours. Furthermore, the result of incubation was diluted with NaCl 0.9% to the same clarity with Mc Farland standard of 10^8 CFU/mL in order to obtain bacterial suspension test.

Antibacterial activity test by cupplate method

Test bacterial suspension was spread on Media for Mueller Hinton (Merck, Darmstat, 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 hours at a temperature of 37ºC. DMSO 10% was used as negative control then Chloramphenicol as positive control. Sterile zone formed is measured in diameter and is called inhibition zone diameter.

Detection of antibacterial TLC spot by bioautography assay

A total of 1.25 mg of extract dissolved in methanol, and then spotted into TLC plate silica gel F 254 (Merck, Darmstat, Germany) and eluted with a mobile phase of chloroform - methanol (7: 3 v/v). The detection is 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 media order in a petri dish, and the results of the TLC plate is dried and affixed to the media for 30 minutes. After TLC plate was reinstated, petri dish is resealed and incubated for 18-24 hours at temperature of 37ºC. Areas that showed 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 FTIR spectrophotometer (Shimadzu, Japan) in KBr disk at wavenumbers of 4000 – 400 cm^{-1}. The measurement is performed in controlled room temperature (20ºC). Characterization of the fraction was also conducted using UV spectroscopy (Shimadzu, Japan), HPLC (Shimadzu, Japan), TLC Scanner (Camag 4, Germany) 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 have been used in determining the species diversity of microorganisms [17]. Therefore, in this study, the results of 16S rRNA gene sequencing was 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 was elected to the data base of existing microorganisms.

Prior to sequencing, first performed PCR to isolate DNA using PCR Primer Lyophilized primer Primer Forward 27F (specific for Bacteria) (5'-AGAGTTTGATCCTGGCTCAG-3 ') and R1492 (5' -TACGG [A / T / C] TACCTTGTTACGACTT- 3 '). PCR was performed in conditions: - pradenaturasi: 3 minutes at a temperature of 96ºC, - 30 cycles of PCR (denaturation: 1 minute at a temperature of 94ºC, anealling: 1 minute at a temperature of 53ºC, extension: 5 min at 72 ° C, - the extension finals: 5 minutes at a temperature of 72ºC. According to the BLAST analysis of the 16S rRNA gene, J4 isolate has a close sequence with Burkholderia sp with identical percentage of 99%. One of these species are *Burkholderia (Pseudomonas) cepacia* that produce phenylpyrrole, a broad-spectrum antimicrobial compound called pyrrolnitrin [3-chloro-4- (2'-nitro-3'-chloro-phenyl) Pyrrole] with the chemical formula C10H6Cl2N2O2 [18]. 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 [19, 20]. 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 with10 % DMSO shows 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 [21,22]. In this study, the comparison Rf values of patches which have activity against *S. aureus* or *E. coli* as well as the positive control (chloramphenicol 0.675 mg) is depicted in Figure 1. The active zone appeared at Rf 0.76 against both *S. aureus* and *E. coli.*

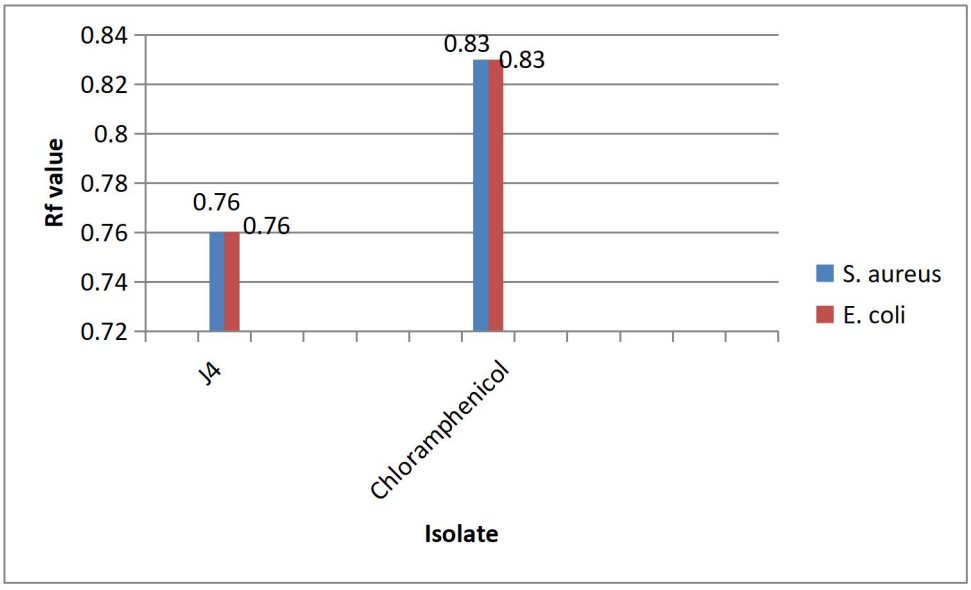

Fig. 1: The active zone of J4 extract against *S. aureus* or *E. coli* was at Rf 0.76, while Chloramphenicol as a positive control was at Rf 0.83

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 fraction CM) 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 fraction CM was only detected 1 spot under 366 nm but

there was no spot under 254 nm. It can be pointed that the fractionation process was well conducted because many metabolites could be eliminated.

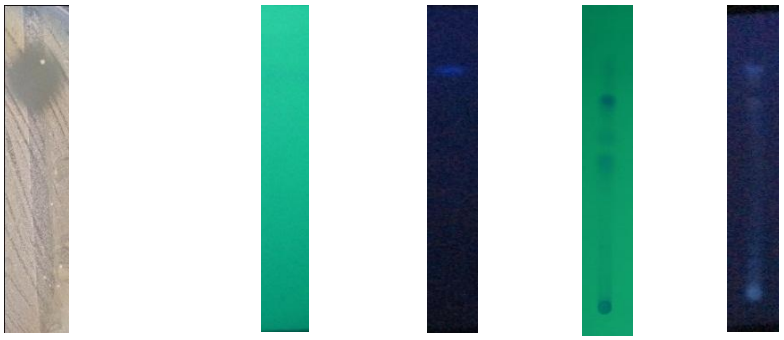

Fig 2 : The active zone of TLC bioautografi of CM fraction (A) and the TLC chromatogram of CM fraction under UV light 254 nm (B) and 366 nm (C) as well as extract under UV light 254 nm (D) and 366 nm (E)

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 HPLC analysis of the active fraction J4 shows that the fraction is not a pure fraction. Although there was only one peak when using 100-70% of methanol, but in the use of the 60% and 50% methanol, it appeared that there was more than 1 peak. The separation profile in the use of methanol 60% as mobile phase is shown in Figure 3 and Table 1. This fraction also produced two major peaks at RT of 2.5 and 2.8 minutes with % area of 31.8% and 60.1%, respectively. In addition, it is also detected two peaks with low intensity with retention times of 1.9 and 5.5 minutes. The two major peaks are at adjacent retention times, namely at minutes 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%.

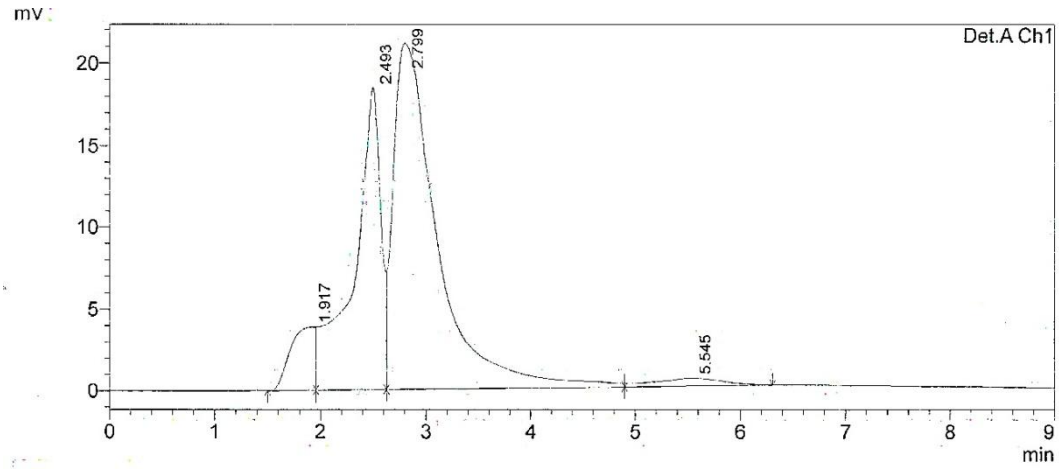

Fig. 3: Separation profile of CM fraction by HPLC in the use of methanol 60% as the mobile phase

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 was using IR Spectroscopy. The FTIR spectrum fraction CM (Fig 4) showed OH stretching vibration frequency in the wavenumbers of 3300 to 3500 cm^{-1} in which 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}.

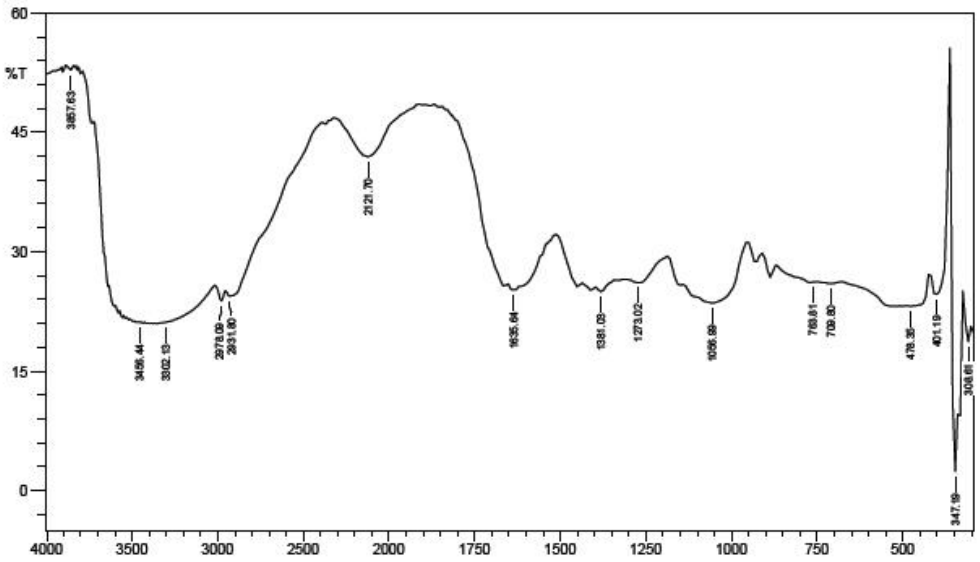

Fig. 4: IR Spectra of CM fraction at wavenumbers of 4,000-400 cm^{-1}

Furthermore, LC-TOF-MS analysis of the fraction CM was used to estimate the chemical formula of the components contained in the fraction. The LC-TOF-MS Chromatogram of the fraction CM is shown in Figure 5 and the m/z data of each chemical formulas are summarized in Table 2. There were 3 peaks detected in the fraction CM, 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.

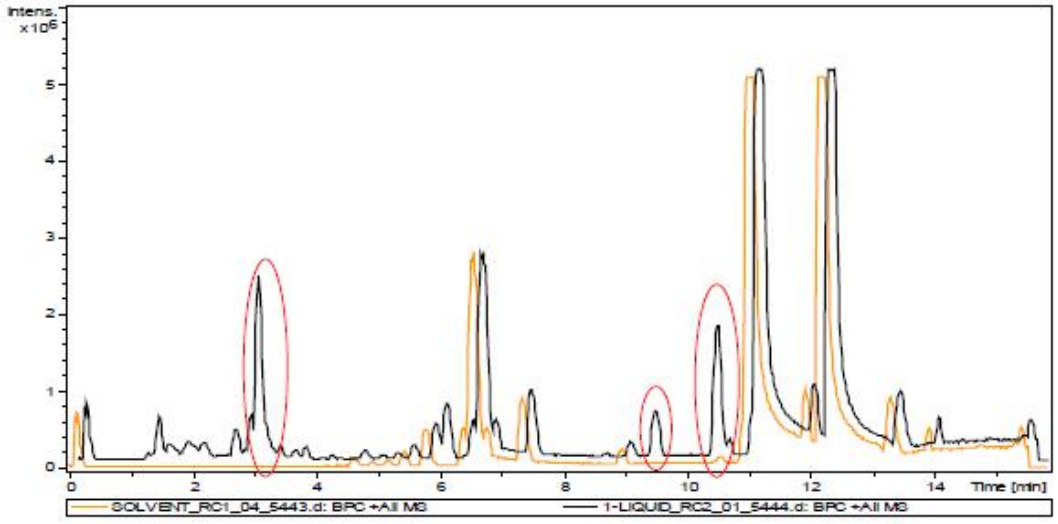

Fig. 4: LC-TOF-MS Chromatogram of CM Fraction. The oval circle shows the peaks of the compound contained in the CM fraction.

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	$C_6H_{12}N_{12}$	253,1	-3,7	9,2
2	9,34	$C_{21}H_{29}N_3O_5$	404,2	3,6	3
		$C_{21}H_{26}N_2O_5$	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 streching, the molecular formula of $C_6H_{12}N_{12}$ with a retention time of 2,94 minutes and m/z of 253 is not suitable because there is no OH group. Therefore, there are 4 possible molecular formulas which are active substances in J4 bacteria, namely C21H29N3O5, C21H26N2O5, C17H22N8O3 or C15H35N3O. These molecular formulas differs from the molecular formulas of pyrrolnitrin, cepacin A and cepacin B, the previously discovered antimicrobial compounds produced by Burkholderia sp [18,20].

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. This formula needs to be determined the real molecular structure 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 (Saltwater Nutrient Agar), SNB (Starch Nitrate Broth), TLC (Thin Layer Chromatography), DMSO, FTIR, ODS,

ACKNOWLEDGEMENT

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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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Artikel :

ANTIBACTERIAL SUBSTANCE PRODUCED BY A SOIL BACTERIA ISOLATED FROM RHIZOSPHERE OF *Zingiber officinale*

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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 its isolates has been known had a metabolite that has 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. Extrction of the culture broth of J4 isolate using ethyl acetate followed with fractionation. 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 its IR spectroscopy examination, there were OH, CH, and carbonyl stretching. LC-TOF-MS analysis showed 5 molecular formulas with m/z of 270, 274, and 404 in the active fraction, but there was one formula with no OH groups.
Conclusion: J4 isolate is a *Burkholderia* sp. The active substances molecular formulas produced by J4 isolate are C21H29N3O5, C21H26N2O5, C17H22N8O3, or C15H35N3O.

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

INTRODUCTION

Antibacterial substance is secondary metabolites produced primarily by microbes living in the soil. Most of these microbials 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 source and to identify the active compounds present.

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) were very useful for chemical compound identification [5,7-9]. Cremin & 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]. Each compound gave a specific spectrum so that there are no two different compounds that have the same spectrum. This is because the frequency of vibration is influenced by environmental bonds [11]. Some advantages of using IR spectroscopy can analyze almost any compound, much of the information obtained from the spectra, relatively quick, easy, and inexpensive. Besides, IR spectroscopy is sensitive because it can analyze the small sample size [12].

Identification of microbial producers of antibacterial active compound can be done by analyzing the diversity of microbial isolates by 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 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 days 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 mM NaCl (Sigma, Aldrich, USA), 25 mM EDTA (Sigma, Aldrich, USA), 20 mM 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). Temperature PCR was set as follows: initial temperature is 80°C for 5 min at, then 30 cycles for 0.5 min at a temperature of 94°C, 0.5 min at 55°C, 1 min 72°C and the final temperature was set at 72°C for 7 min. 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

A total of 2 plug 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 days. A total of 30 mL of liquid culture was transferred into 300 mL medium SNB and incubated 5 days at room temperature, and then transferred again to 2.7 L SNB media and incubated for 14 days at room temperature. Furthermore, the culture fluid is 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 is done successively with hexane, chloroform-methanol (7:3 v/v), and methanol. Each fraction is 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 37ºC. DMSO 10% was used as negative control then Chloramphenicol as a positive control. Sterile zone formed is measured in diameter and is called inhibition zone diameter.

Detection of antibacterial TLC spot by bioautography assay

A total of 1.25 mg of extract 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 is 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 media order in a petri dish, and the results of the TLC plate is dried and affixed to the media for 30 min. After the TLC plate was reinstated, petri dish is resealed and incubated for 18-24 h at a temperature of 37ºC. 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 is performed in controlled room temperature (20ºC). 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 have 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.

Before sequencing, first performed PCR to isolate DNA using PCR Primer Lyophilized primer Primer Forward 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 96ºC, - 30 cycles of PCR (denaturation: 1 min at a temperature of 94ºC, annealing: 1 min at a temperature of 53ºC, extension: 5 min at 72 ° C, - the extension finals: 5 min at a temperature of 72ºC. 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 shows 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 Figure 1. The active zone appeared at Rf 0.76 against both *S. aureus* and *E. coli.*

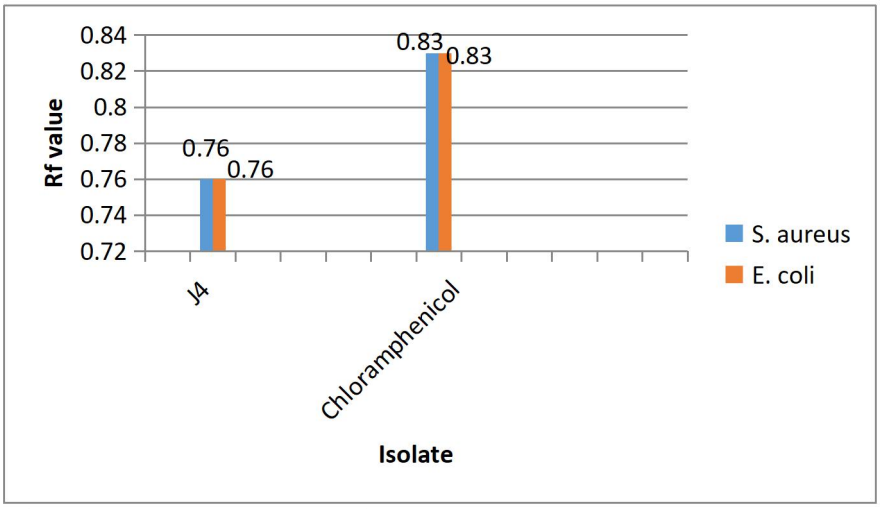


Fig 1: The active zone of J4 extract against *S. aureus* or *E. coli* was at Rf 0.76; while Chloramphenicol as a positive control was at Rf 0.83

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 fraction CM) 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 fraction CM 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.

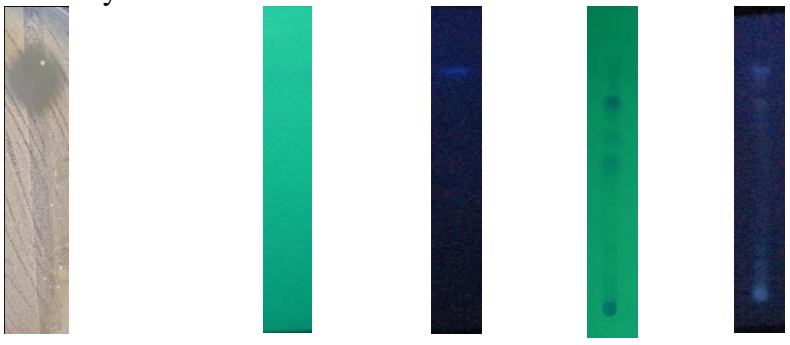
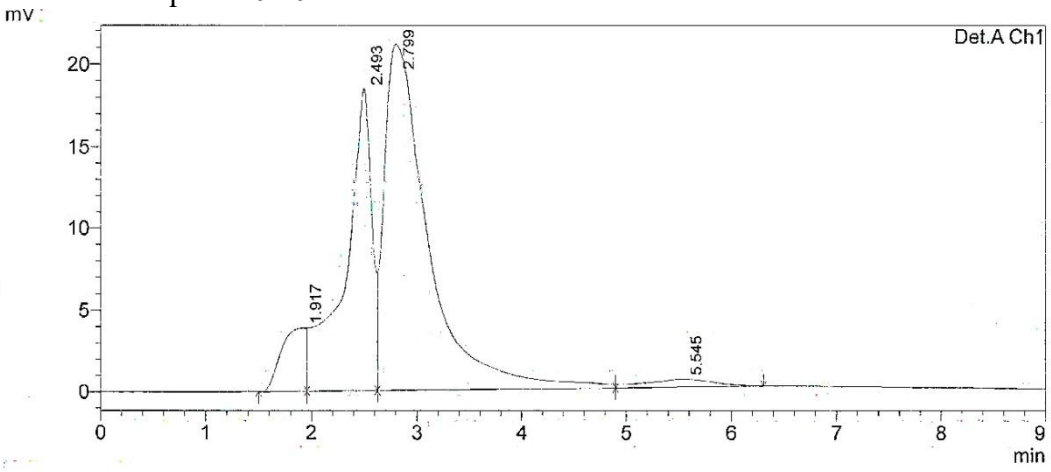


Fig 2: The active zone of TLC bioautography of CM fraction (A) and the TLC chromatogram of CM fraction under UV light 254 nm (B) and 366 nm (C) as well as extract under UV light 254 nm (D) and 366 nm (E)

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 Figure 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 a 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 was using IR Spectroscopy. The FTIR spectrum fraction CM (Fig 4) showed OH stretching vibration frequency in the wavenumbers 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 wavenumbers 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}.

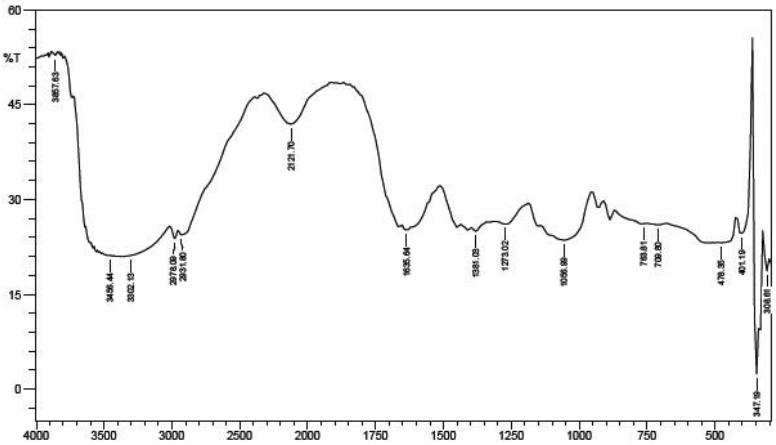


Fig 4: IR Spectra of fraction at CM wavenumbers of 4,000-400 cm^{-1}

Furthermore, LC-TOF-MS analysis of the fraction CM was used to estimate the chemical formula of the components contained in the fraction. The LC-TOF-MS Chromatogram of the fraction CM is shown in Figure 5 and the m/z data of each chemical formulas are summarized in Table 2. There were 3 peaks detected in the fraction CM, 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.

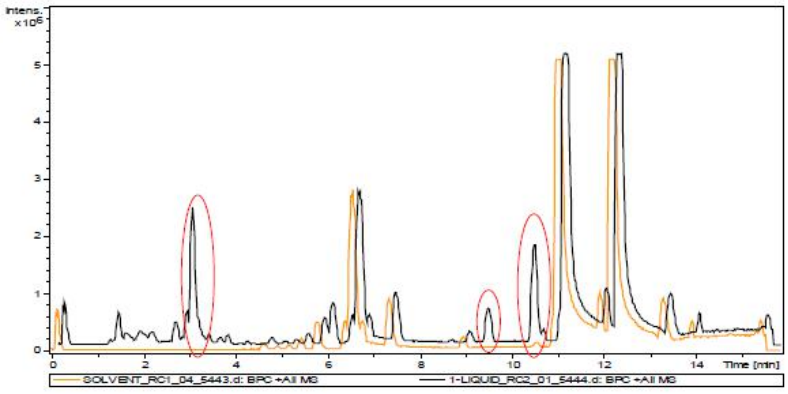


Fig 4: LC-TOF-MS Chromatogram of CM Fraction. The oval circle shows the peaks of the compound contained in the CM fraction.

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	$C_6H_{12}N_{12}$	253.1	-3.7	9.2
2	9.34	$C_{21}H_{29}N_3O_5$	404.2	3.6	3
		$C_{21}H_{26}N_2O_5$	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 not suitable because there is no OH group. Therefore, there are 4 possible molecular formulas that are active substances in 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 Chromatography), SNA (Saltwater Nutrient 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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Lampiran 4. Email tentang keputusan dari Editor bahwa artikel diterima untuk publikasi di IJAP.

Gmail nanik sulistyani <naniksulistyani@gmail.com>

[IJAP] Your manuscript IJAP 12 Special Issue 3rdPSPS: Acceptance

the3rd psps <the3rdpsps@gmail.com> 9 Oktober 2020 20.33
Kepada: naniksulistyani@gmail.com, jwidada@gmail.com, yosibayu.murti@gmail.com, mustofajogja@yahoo.com

Dear Nanik Sulistyani, Jaka Widada, Yosi Bayu Murti, and Mustofa

I am happy to inform you regarding your submission to the International Journal of Applied Pharmaceutics through The 3rd PSPS 2020 entitled " Antibacterial substance produced by a soil bacteria isolated from rhizosphere of zingiber officinale" that it has been recommended for publication after peer review.

Your article is now accepted for publication and your article is scheduled to be published in Vol 13, Special Issue 1, Jan 2021.

Please find in the attachment your payment receipt and letter of acceptance.

Best Regards,
The 3rd PSPS Committee

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ACCEPTANCE LETTER

Ref: Special issue 1 (3rd PSPS) Date: 09/10/2020

To,

The Committee of The 3rd Postgraduate Seminar on Pharmaceutical Science 2020

Dear
Sir/Madam,

I am pleased to inform you that the following articles (Listed below) have been accepted for publication in IJAP (subjected to receipt of publication fee). These are scheduled to be published in special issue: Vol 13, Special Issue 1, Jan 2021.

No	Title	Author	Email
1	Antioxidant and antihyperglycemic potential of klutuk banana peel fraction (*Musa balbisiana* Colla)	Tita Nofianti M.Si., Apt Ahmad Muhtadi Irda Fidrianny Ai Samrotul Fuadah Vera Nurviana Ruswanto	titanofianti.wamsu@gmail.com a.muhtadi@unpad.ac.id irdafidrianny@gmail.com aisamrotulf@gmail.com veranurviana@stikes-bth.ac.id ruswanto@stikes-bth.ac.id
2	Formula optimization and in vitro release kinetic studies of diltiazem hydrochloride mucoadhesive bilayer buccal film	Lina Winarti Afalah Zulfa Laily Lusia Oktora RKSE Deddy Irawan Lidya Ameliana Kuni Zuaimah Barikah Viddy Agustian Rosyidi Dwi Nurrahmanto	lina.winarti@unej.ac.id afalahzulfal@gmail.com oktora@unej.ac.id eka.deddyi@unej.ac.id lidyaameliana@unej.ac.id kuni.farmasi@unej.ac.id viddy.farmasi@unej.ac.id dwinurahmanto.farmasi@unej.ac.id
3	Ethanol recovery from propolis production waste using adsorption distillation method	Muhamad Sahlan Lindatiana Yulistiono Ashadi Sasongko Apriliana Cahya Khayreani Kenny Lischer Diah Kartika Pratami	sahlan@che.ui.ac.id lindatianayulistiono@gmail.com ashadisasongko@lecturer.itk.ac.id apriliana.cahya@ui.ac.id lischer.kenny@ui.ac.id d.kartika@univpancasila.ac.id
4	Development of CODI (Co-drug interaction) software as drugs prescription analysis	Muhamad Rinaldhi Tandah Yusriadi Alwiyah Mukaddas Khusnul Diana Ahmad Anggara Sadewa	prof.aldhi@gmail.com yuseryuser@gmail.com; alwiyah.mukaddas@gmail.com cuz.aldhi@gmail.com anggarasadewa13@gmail.com