KORESPONDENSI PAPER

JUDUL : **Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus***

JURNAL **: Jordan Journal of Biological Sciences (JJBS)**

No	Aktivitas	Tanggal	Keterangan	Halaman
1	Manuscript submission	4 Desember 2020	Email: Submit ke Redaksi JJBS • Manuskrip yang di-submit • Cover letter • Copyright	2 3-16 17 18
2	Received manuscript	10 Desember 2020	Email: Manuscript ID: JJBS 326/20 & permintaan Turnitin(<15%)	19
3	Submit Turnitin	11 Desember 2020	Email: Hasil Turnitin ID: JJBS 326/20	20
4	Evaluation manuscript	19 Pebruari 2021	Email: JJBS MS 326/20 (Evaluation) • Evaluation Report- Referee 1 • Evaluation Report- Referee 2	21 22-23 24-25
5	Manuscript sent for Review #1	6 Maret 2021	Email: Submit Rev_ Manuscript ID: JJBS 326/20 • Submit Revision File#1	26 27-44
			• Respons to Referee 1 • Respons to Referee 2 • Revised manuscript #1	27 28 29-44
6	Decision by Reviewers	11 Maret 2021	Email: Both reviewers agree revised Manuscript ID: JJBS 326/20 & references correction	45
7	Submit references correction	13 Maret 2021	Email: references in JJBS style • Revised references	46 46-48
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9	Galley proof	20 Pebruari 2022	Email: Galley proof Manuscript ID: JJBS 326/20 • Corrections text	50 50
10	Galley proof response	21 Pebruari 2022	Email: Submit Galley proof Manuscript ID: JJBS 326/20 • Revisions text	51 51
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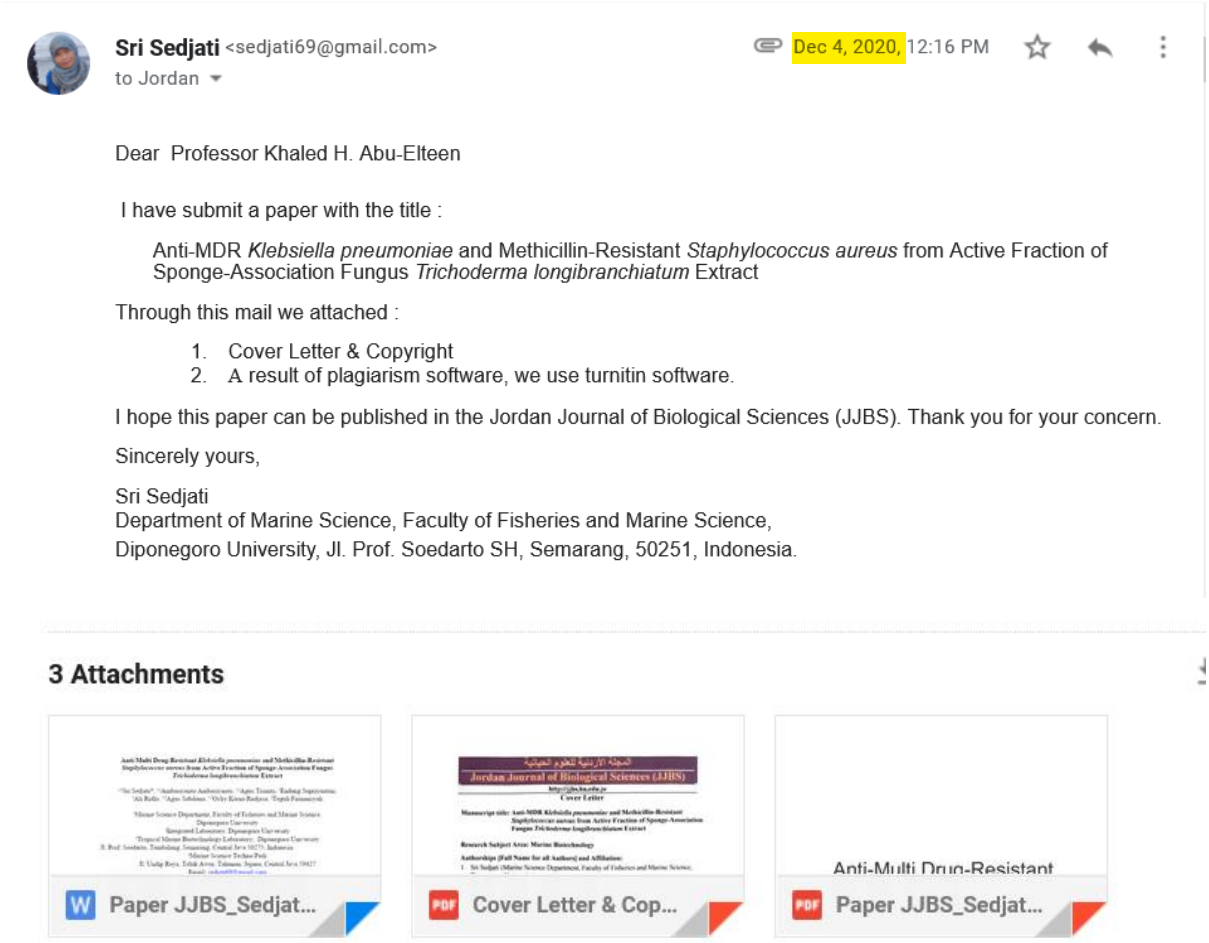

Sri Sedjati <sedjati69@gmail.com>
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Dec 4, 2020, 12:16 PM

Dear Professor Khaled H. Abu-Elteen

I have submit a paper with the title :

Anti-MDR *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibranchiatum* Extract

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3 Attachments

- Manuskrip yang di-submit

Anti-Multi Drug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibrachiatum* Extract

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Abstract

Extracts from sponge association fungus *T. longibranchiatum* has been studied and contains antibacterial compounds which can inhibit several pathogenic multidrug-resistant organisms. This study aims to determine the active fraction of the extract which is antibacterial against the gram-negative MDR *K. pneumoniae* pathogen and gram-positive MRSA. In this study, the fungus was cultivated using solid media of malt extract agar (MEA) for 6-9 days (24 hours dark, static, pH 5.6, 60 ‰ salinity, and 27 ^{o}C). The mycelia and media were macerated by methanol and then partitioned using ethyl acetate. Active fraction tracing was carried out using the bioautography method and then isolated by open column chromatography method. Antibacterial activity testing was done using the Broth Dilution method to determine the Minimum Inhibitory Concentration (MIC). The results of the study showed that ethyl acetate extract contained one active fraction (R_f value = 0.14), which has reactive characteristics on vanillin reagent and absorbed ultraviolet light (λ 375.5 nm absorbance peak). The active fraction was able to inhibit the growth of MDR *K. pneumoniae* and MRSA bacteria at the same MIC value, i.e. 256 µg mL^{-1}. In conclusion, an active fraction of *T. longibranchiatum* can be developed as an antibacterial against MDR *K. pneumoniae* and MRSA.

Keywords: *Trichoderma longibranchiatum*, active fraction, antibacterial, *Klebsiella pneumoniae*, *Staphylococcus aureus*

1. Introduction

K. pneumoniae is an opportunistic pathogen, can be categorized in gram-negative group bacteria which has thick, non-motile, facultatively anaerobic, and rod-shaped cell walls. These bacteria produce Extended Spectrum β-Lactamases (ESBL) which can degrade certain antibiotics (β-lactam group), such as penicillin and cephalosporin, so they become inactive (Ullah *et al*., 2009; Bimanand *et al*., 2017). Also, it is protected by a capsule (composed of

polysaccharides), both of which will further increase its pathogenicity. The infectious diseases caused by them are such as liver abscess, bacteremia, lung infection, acute leukemia, meningitis, and the bacteria may even cause death (Turton *et al*., 2010). Hospitalized patients with weak immunity will be the main target for this bacterial attack. In current conditions, there is a tendency to increase the prevalence of infection caused by *K. pneumonia* along with a decrease in sensitivity to antibiotics used to treat the infection (Li *et al.*, 2014; Santana *et al*., 2016).

S. aureus (gram-positive bacteria) is another cause of some dangerous infection. The characteristics of these bacteria: coccus-shaped, non-spore-forming, non-motile, facultative anaerobes, and form biofilm. In particular, biofilm formation on MRSA infection make difficult treatment and causes a hard prognosis (Sato *et al*., 2019). For a long time, the infection has been treated by such semi-synthetic penicillin antibiotics as methicillin, and it was resistantly used in 1961s (Gajdács, 2019). MRSA incidence (prevalence) is more likely to occur in health care/hospital settings area than in the community environment. These bacteria produce enterotoxins (exotoxins), ESBL enzymes, and immune modulators (Abbas *et al*., 2015).

According to Narendran and Kathiresan (2016) as well as Basiriya *et al.* (2017), some species of *Tricoderma* sp. have been researched and eventually have the ability to synthesis some antibacterial compounds. Moreover, some studies reported that ethyl acetate extract of *Trichoderma* sp. to be antibacterial against pathogens (*Pseudomonas. aeruginosa, Escherichia coli, Bacillus cereus,* and *S. aureus,*). These fungi, isolated from the mangrove rhizosphere, were proven to produce secondary metabolites that can be developed become a new antibiotic against resistant bacteria. This was also stated by Synytsya *et al.* (2017) who investigated antibacterial compounds derived from ethanol and petroleum ether extracts. Some other researchers stated that the potential of antibacterial compounds produced by *Trichoderma* sp., such as *trichodin* A and B, pyridoxatin is antibacterial against *S. epidermidis* and *S. aureus* (Wu *et al.*, 2014; Wang *et al*., 2020), and trichodermaquinone to be antibacterial against MRSA (Khamthong *et al*., 2012). There are many classes of secondary metabolite as antibacterial compounds from marine fungi, such as glycopeptides, peptides, proteins, lipopeptides, aminolipopeptides, polyketides, polybrominated biphenyl ether, cyclic depsipeptides, terpenes, pentaketides, alkaloids, dicetopiperazins, anthraquinones, chromones, steroids, lactones, quinolone derivatives, trisindole derivatives, macrolactam, and phenol derivatives (Thomas *et al*., 2010; Nalini *et al*., 2018; Wang *et al*., 2020).

The results of the review by Li *et al.* (2019) showed that *Trichoderma* spp. are the producer of many secondary metabolites with different biological activities. These fungi are commonly distributed in many ecosystems, including the sea. The investigation by Sedjati *et al.* (2020) proved that ethyl acetate extract of sponge-association fungus *T. longubranchiatum* contains antibacterial compounds against MRSA and *K. pneumoniae*. Based on these findings, this study aims to determine the active fraction in the ethyl acetate extract of *T. longubranchiatum* using autobiographical methods by the guidelines of its bioactivity test results.

2. Material and Methods

2.1. Fungus Isolate

The samples used in the study were the results of fungi obtained from Falajava Beach, Ternate Island, North Maluku, Indonesia (00^{o}47'09.12" N; 127^{o}23'21.76" E coordinates) with TE-PF-03.1 code. The fungi have been identified molecularly using ITS rDNA sequence, and macro and microscopically confirmed as *T. longibrachiatum* (Sedjati *et al*., 2020).

2.2. Bacterial Pathogen

The test bacteria used in this experimental study were MDR *K. pneumoniae* from Microbiology Laboratory, Diponegoro National Hospital, and MRSA from the University of Indonesia (UI). Pathogenic bacteria were recultured being used for antibacterial testing. The process was done by taking bacterial stock colonies and transferring them into Nutrient Broth (NB) and further incubated for 24 hours at 37^{o}C temperature.

2.3. Fungus Cultivation

T. longibranchiatum isolate coded TE-PF-03.1 was refreshed using Malt Extract Agar (Merck's MEA) media on a petri dish (20 mL media/petri). MEA preparation was conducted by using sterile seawater with 48 g in 1 L dose. The final composition of MEA consisted of malt extract (3%), peptone (0,3%), and agar (1,5%). After 7 days of incubation, the mycelia were taken using a circular loop needle with approximately 2 mm in diameter and cultivated on new MEA media for 6-9 days (24 hours in dark, static, pH 5.6, 60‰ salinity, and 27^{o}C temperature).

2.4. Extraction and Determining Extract Weight

After the cultivation period has finished, the media and the micelle were cut into small pieces and then macerated with methanol (1:1v/v), filtered using Whatman paper no. 42 and the filtrate was evaporated at a rotary evaporator with 40^{o}C and low pressure. Furthermore, the

extract was partitioned using methanol-distilled water (70%) and ethyl acetate (1:1v/v). Moreover, each fraction was evaporated using rotavapour to be methanol and ethyl acetate extracts and then these were weighed.

2.5. *Profiling* Metabolit Sekunder

The Thin Layer Chromatography (TLC) method (Harborne, 1984) was used for profiling chemical extracts. There were in total 10 µl of extract solution in ethyl acetate or methanol (1 mg/ml) was spotted on the baseline of the TLC plate. Several mobile phases (n-hexane, ethyl acetate, chloroform, acetone, and methanol) with various comparisons were experimented with to elute the extracts until obtaining a completely separated spot profile. The mobile phases used were sequential based on polarity levels, i.e. a mixture of n-hexane and ethyl acetate (4:1; 3:2; 2:3; 1:4, and 0:5). Spot identification using the value of R_f (Retention Factor) and spraying with staining reagents. After the elution process, TLC was visualized by UV light (365 nm) and then sprayed with 2% vanillin-H_2SO_4 reagent, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (Harborne, 1984; Sen *et al*., 2012; Trianto *et al* .., 2019). TLC plate was furthermore heated at 110 ^oC temperature for 2-3 minutes. The same method was used to detect the active antibacterial fraction after the isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

2.6. Antibacterial Activity Test

The antibacterial bioassay was conducted by determining the diameter of the inhibition zone using a disc diffusion assay (Trianto *et al*., 2017). Pathogenic bacteria were cultured in Mueller-Hinton Agar (MHA) medium. Besides, the preparation used a bacterial suspension in 24 hours aged MHB with 0.5 McFarland (1.5×10^8 CFU mL^{-1}) density which was then inoculated by swab method and left it still for 15 minutes to ensure all preparats were absorbed by the media. Extracts preparation was started by making a solution of stock with 50 mg mL^{-1} concentration in dimethyl sulfoxide (DMSO). 10 µL of extract solution was dropped onto the surface of sterile disc paper (Oxoid, 6 mm diameter) resulting in 500 µg/disc on final concentration. Each extract in the DMSO solvent was tested against pathogenic bacteria. The positive control used chloramphenicol (Oxoid, 30µg/disc). Meanwhile, the inhibition zone was measured after 24 hours incubation period at 37 ^oC temperature.

2.5. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured

in MHA media with 1.5 x10^8 CFU mL^{-1} initial density. The extract in ethyl acetate solvent (10 µL, 10 mg mL^{-1} concentration) was spotted on the TLC surface baseline and eluted with a suitable mobile phase to produce perfectly separated spots. TLC plate was applied with silica surface attached to the MHA media surface (facing downward) and left for 60 minutes. Furthermore, the TLC plate was removed from the test bacteria medium and the petri dishes were closed. All processes were carried out in a laminar flow cabinet. The incubation was carried out for 24 hours at 37 ^oC temperature and the formation of the inhibition zone around the TLC spot was carefully observed. The spots around which the next inhibition zone appears were called an active fraction.

2.6. Active Fraction Isolation

The active fractions found were isolated using the open column chromatography (OCC) method with the appropriate mobile phase (referring to the TLC profile). The column was filled with silica gel (60–120 mesh) mixed with n-hexane: ethyl acetate (1: 1) solvent. The extract was dissolved in the solvent and was slowly being loaded on the top surface of the silica gel. The extract was then eluted using a solvent sequence based on the polarity increase. Furthermore, the eluate coming out was collected using a test tube (every 10 mL of eluate), checked again using the TLC method, and the same eluates were put together. The eluates containing active fraction were concentrated at a rotary evaporator for further testing.

2.7. Minimum Inhibitory Concentration Test

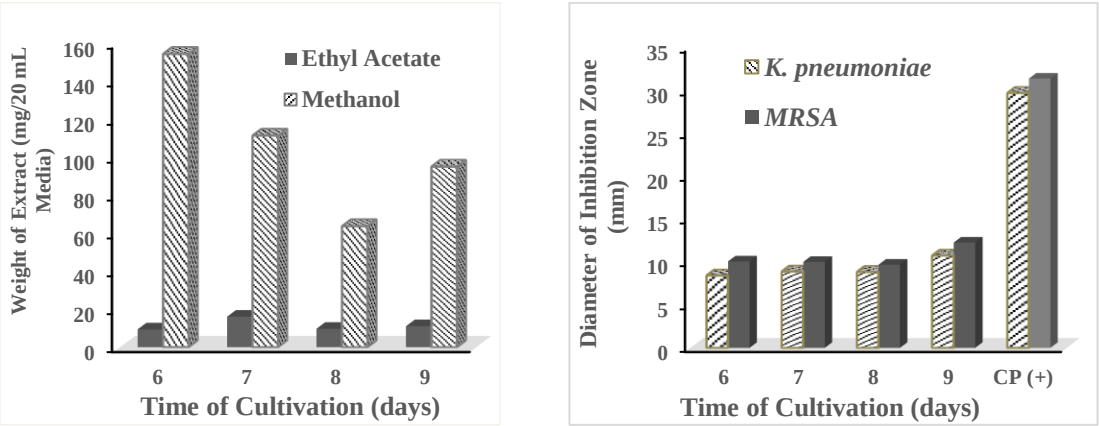
Minimum Inhibitory Concentration (MIC) test based was conducted based on the Broth Dilution method. MIC determination refers to the method proposed by Sowjanya *et al.* (2015) and Fajarningsih *et al.* (2018) using 96-well microplates with resazurin (Sigma-Aldrich) as an indicator of the viability of the test bacteria (REMA assay*)*. A total of 100 µL of the extract solution in the DMSO solvent with the highest concentration (2,048 µg mL^{-1}) was filled in the first well in certain rows. The next well was filled with 50 µL of sterile MHB nutrients. The 50 µL test material was transferred from the first well to the next well to reach serial dilution (at wells No. 1-11). Then, 30 µl of resazurin solution (0.02% in distilled water) was added to each well. At last, 10 µl of the bacterial suspension (1x10^8 CFU mL^{-1}) was added to each well. Chloramphenicol was used as a positive control (the highest concentration was at 64 µg mL^{-1}). Growth control was made in the 12^{th} row, the well contained only MHB growth media without the addition of test material. The microplate was incubated at 37 ^oC for 24 hours. After the incubation period, the well functioning as growth control would appear pink. The MIC value

was determined based on the lowest concentration which could inhibit the growth of the tested bacteria and it appeared blue.

3. Results and Discussion

3.1. Assessment of Antibacterial Potential

T. longibranchiatum species is one of the fungi species which are easy to cultivate; can grow well in MEA media and can be modified using water infusion of fish extracts, cassava, tempeh, and tofu. All of those can produce secondary metabolites, but only the metabolites obtained from cultivation using MEA produce the best antibacterial activity. The best potential reached is its antibacterial properties against *K. pneumoniae* and MRSA (Sedjati *et al*., 2020). Data from the results of this study indicated that the secondary metabolite product of *T. longibranchiatum* cultivated in MEA was mostly in the form of methanol extract (polar compound) and only a small part of them was ethyl acetate extract (semi-polar to non-polar compound). The weight of fungal extract based on the polarity of its secondary metabolites after 6-9 days cultivation periods described in Figure 1a. Ethyl acetate extract was produced in only small amounts. On the other hand, the ethyl acetate extract has antibacterial activity against these two pathogens as shown in Figure 1b. The greatest potential as an antibacterial was seen in the ethyl acetate extract from the fungus which had been cultivated for 9 days.



(a) (b)

Figure 1. Characteristics of *T. longibranchiatum Extract*: (a) Extract Weight on Polarity basis; (b) Antibacterial Potential of Ethyl Acetate Extract Against MDR Pathogens *K. pneumoniae* and MRSA

Some synthesized fungal secondary metabolites are only in small amounts because they are not for the main energy supply needed by the fungus and are only made at suboptimal conditions as a response to environmental pressure (Nielsen and Nielsen, 2017). The peak of secondary metabolite production in this study occurs when the fungal life cycle was in a stationary phase. This statement conforms to several research results stating that the production of secondary metabolites will increase when entering the stationary phase and the fungus has entered a stationary period on day 6 to 9 after being cultivated (Gliseida *et al.*, 2013; Arumugam *et al.*, 2015). Polar secondary metabolites seemed to predominate over the extract of *T. longibranciatum.* However, when tested for antagonists against *K. pneumoniae* and MRSA at a concentration of 500 µg/disc, they did not show antibacterial activity. This fact is similar to the research result of Leylaie and Zafari (2018). In general, the ethyl acetate extract metabolite of the *T. longbranchiatum* is more likely to be antibacterial than its methanol extract. According to the statement of Chamekh *et al.* (2019), methanol extract is presumed to contain enzymes synthesized by *T. longibranchiatum* for external digestion, along with several units of saccharides, amines/peptides, fatty acids/glycerol which are hydrolysis results of organic compounds in the media. Polar metabolites dissolved in methanol consists of enzymes (such as amylase, protease, and lipase) which are synthesized by fungi to degrade the nutrients in the media.

3.2. Secondary Metabolite of Fungal Extract

Chemical compounds contained in ethyl acetate extract of *T. longibranciatum* which was cultivated for 9 days can be traced based on its TLC profile as shown in Figure 2. Only ethyl acetate extract was used for the next stage of research since methanol extract is not potentially as antibacterial. The best spot separation was seen in the results of TLC with a mobile phase with a 2:3 ratio as seen in Figure 2 (3^{rd} order). Based on the number of spots that appeared, at least 5 compounds were detected with R_f values: 0.14, 0.26, 0.57, 0.71, and 0.89. As congenial with the order of R_f values, the compound with the smallest R_f value is relatively the most polar and the largest R_f is the most non-polar.

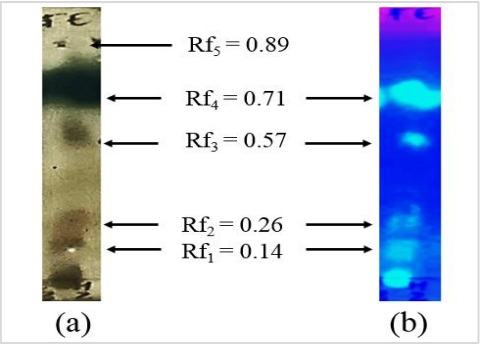
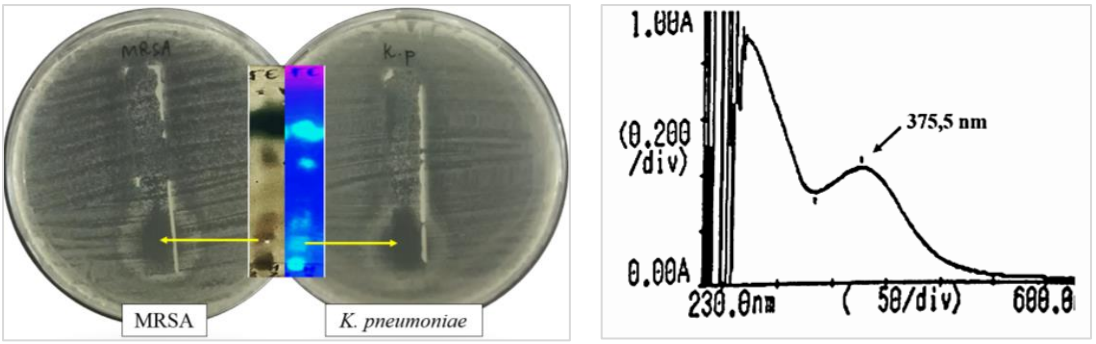


Figure 2. TLC Profile of Ethyl Acetate Extract Using Mobile Phase of n-Hexane and Ethyl Acetate (2:3) : (a) Visualization Results with 365 nm UV Light; (b) Visualization Results with Vanilli-H2SO4 1%

Compound prediction in ethyl acetate extract of *T. longibranchiatum* was traced based on previous studies' references. The spot looks fluorescent blue when exposed to UV indicating that the organic compound has a double bond (polyene or conjugated compound). The increased wavelength of the UVs (200-400 nm) absorbed indicates that the number of double bonds also increased (Hamilton-Miller, 1973; Mohammed, 2018). Besides, compounds reacting positively with the vanillin reagent will appear in certain colored spots (varied colors) characterizing the presence of carbonyl functional groups that contain a carbon-oxygen double bond (aldehydes, ketones). Accordingly, these compounds probably are from terpenoids, fatty acids/essential oils, steroids, flavonoids, or phenolic groups. A compound that reacts negatively to ninhydrin shows that it is not a nitrogen compound or its derivative. In contrast, negative to ferric (III) chloride indicates that the compound does not have a phenol functional group (Harborne, 1984; Jork, 1990). In this study, several spots in the TLC profile of ethyl acetate extract reacted positively to 365 nm UV light and vanillin reagent, but all of them reacted negatively to ninhydrin and ferric (III) chloride (as shown in Figure 2).

3.3. Active Fraction as Antibacterial Against *K. pneumoniae* and MRSA

After an autobiography test was conducted on *K. pneumoniae* and MRSA pathogens, it was found that the spot with the smallest R_f (0.14) was the active fraction as antibacterial. The results of the autobiography test will help detect the presence of antibacterial compounds by the formation of an inhibition zone around the active spot as shown in Figure 3.



(a) (b)

Figure 3. Determination of Active Fraction as Antibacterial **:** (a) Autobiographical Results of Ethyl Acetate Extract Against MRSA and MDR *K. pneumoniae*, (b) Characteristics of Antibacterial Active Fraction Based on Spectra Patterns towards UV Light Absorbance

The active fraction was reactive to vanillin and 365 nm UV light on TLC visualization results and was strengthened by the active fraction spectra pattern using a UV-Vis spectrophotometer which has λ 375.5 nm absorption peak (illustrated in Figure 4). Based on the description of this characteristic, the active fraction is likely thought to have a carbonyl group and contain conjugated double bonds.

3.4. Minimum Inhibitory Concentration of Active Fraction

Antibacterial activity of the active fraction against MDR *K. pneumoniae* and MRSA pathogens resulted in a similar MIC value, i.e. at 256 μg mL^{-1}. *K. pneumoniae* bacteria are categorized as gram-negative bacteria, while MRSA is gram-positive. Both bacteria are still sensitive to chloramphenicol antibacterial since their MIC value is less than 8 μg mL^{-1}.

Table 1. MIC Value of Active Antibacterial Fraction and Chloramphenicol Against MDR *K. pneumoniae* and MRSA Pathogens

Test Material	Value of MIC (μg mL^{-1}) against Pathogens	
	MRSA	*K. pneumoniae*
Active Fraction	256	256
Chloramphenicol	4*	4*

*MIC>32 = resistant, 18-32 =intermediate, <8 =sensitive (CLSI, 2017)

The cell wall of gram-negative bacteria is thicker, composed of peptidoglycan, 2 layers of phospholipids, and is protected by a lipopolysaccharide capsule. Gram-positive bacteria have thinner walls composed of peptidoglycan and lipoteichoic acid, and 1 layer of phospholipids

(Lima *et al*., 2013). Both bacteria are still sensitive to chloramphenicol antibacterial since their MIC value is less than 8 μg mL^{-1} (CLSI, 2017). Chloramphenicol is a commercial broad-spectrum antibacterial. Moreover, chloramphenicol can damage important metabolic pathogens by binding the 50S ribosome subunit and blocking essential ribosomal function. The interaction of nitrobenzyl functional group from chloramphenicol and the bacterial RNA nitrogen base may interfere with the formation of peptides during the process of protein biosynthesis done by bacteria (Kostopoulou *et al*., 2011).

The active fraction resulted from this study had a carbonyl group and also alternating double bonds (conjugation). The aldehyde and ketone carbonyl groups are highly polarized because carbon is less electronegative than oxygen. Carbon contains a partial positive charge (δ^+), while oxygen has a partial negative charge (δ^-). Hence, the carbonyl group can function as a nucleophile and an electrophile. The conjugation of a double bond to the carbonyl group will transmit the electrophilic character of the carbonyl to the beta-carbon of the other double bonds, or popularly called charge delocalization (Sarker and Nahar, 2007). Charged compounds ions will make them easier to interact with bacterial cell wall so that they can penetrate the cytoplasm wall.

The mechanism of action of the active fraction as an antibacterial is assumed to be related to its ability to form electrophile sites, i.e. C with δ^+ partial charge which will electrostatically interact with the phospholipid head (PO_4^-) on the bacterial cell wall. According to Malanovic and Lohner (2016), a positive charge is essential for the initial binding to the surface of the bacterial membrane with a negative charge, which allows it to enter the bacterial cell membrane. Furthermore, these active compounds can affect the metabolic activity of bacterial cells which will eventually cause growth retardation or even death of pathogens.

T. longibrachiatum fungus does not only live in association with sponges in the sea. However, it has also been previously found in soft corals from the waters of Panjang Island, Central Java. In addition, its ethyl acetate extract was able to inhibit the growth of MDR-*S. haemolyticus* and produced a 12.2 mm inhibition zone at a concentration of 300 μg/disc (Sabdaningsih *et al*., 2017). The secondary metabolites from the same fungus has been published by Sperry *et al.* (1998). The ethyl acetate extract of *T. longibrachiatum* is associated with *Haliclona sp.* sponge from Sulawesi water containing an epoxysorbicillinol ($C_{14}H_{16}O_5$), is a member of sorbicillinoids (vertinoids) polyketide compounds. According to Harned and Volp (2011); Meng *et al*. (2016); Salo *et al*. (2016), sorbillinoids are secondary metabolites of hexaketide that are cyclization. Its chemical structure has several double bonds and carbonyl groups. The

results of a study from Corral *et al*. (2018) showed that some of these have antibacterial activity, such as sorbicillin ($C_{14}H_{16}O_3$), sorbicillinol ($C_{14}H_{16}O_4$), dihydrosorbicillin ($C_{14}H_{18}O_4$), oxosorbicillinol ($C_{14}H_{16}O_5$), bisvertinol ($C_{28}H_{34}O_8$), and bisvertinolone ($C_{28}H_{32}O_9$). These compounds can inhibit pathogens *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *S. aureus*, and *K.pneumoniae*.

4. Conclusion

T. longibranchiatum fungi extracts contain an active fraction that can be developed as an antibacterial against gram-negative pathogens *MDR K. pneumoniae* and gram-positive MRSA. The active fraction is assumed to contain a carbonyl functional group and a conjugated double bond. The mechanism of its antibacterial action is related to the formation of electrophile sites on carbon. Thus, electrostatic interactions occur with negative charges on the cell walls of both gram-positive and gram-negative bacteria making it possible to penetrate the cytoplasmic wall. The active fraction of ethyl acetate extract was antibacterial against pathogens MDR *K. pneumoniae* and MRSA with the same MIC value, i.e. 256 μg mL^{-1}

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- Cover letter

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Cover Letter

Manuscript title: Anti-MDR *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibranchiatum* Extract

Research Subject Area: Marine Biotechnology

Authorships [Full Name for all Authors] and Affiliation:

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2. Ambariyanto (Marine Science Department, Faculty of Fisheries and Marine Science, Diponegoro University).
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8. Teguh Firmansyah (Integrated Laboratory, Diponegoro University)

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Signature (on behalf of all co-authors) :

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Manuscript ID: JJBS 326/20 **Dec 10, 2020**

Anti-Multi Drug-Resistant Klebsiella pneumoniae and Methicillin-Resistant Staphylococcus aureus from Active Fraction of Sponge-Association Fungus Trichoderma longibranchiatum Extract

Dear Dr

Sri Sedjati*, Ambariyanto Ambariyanto, Agus Trianto, Endang Supriyantini, Ali Ridlo, Agus Sabdono, Ocky Karna Radjasa, Teguh Firmansyah

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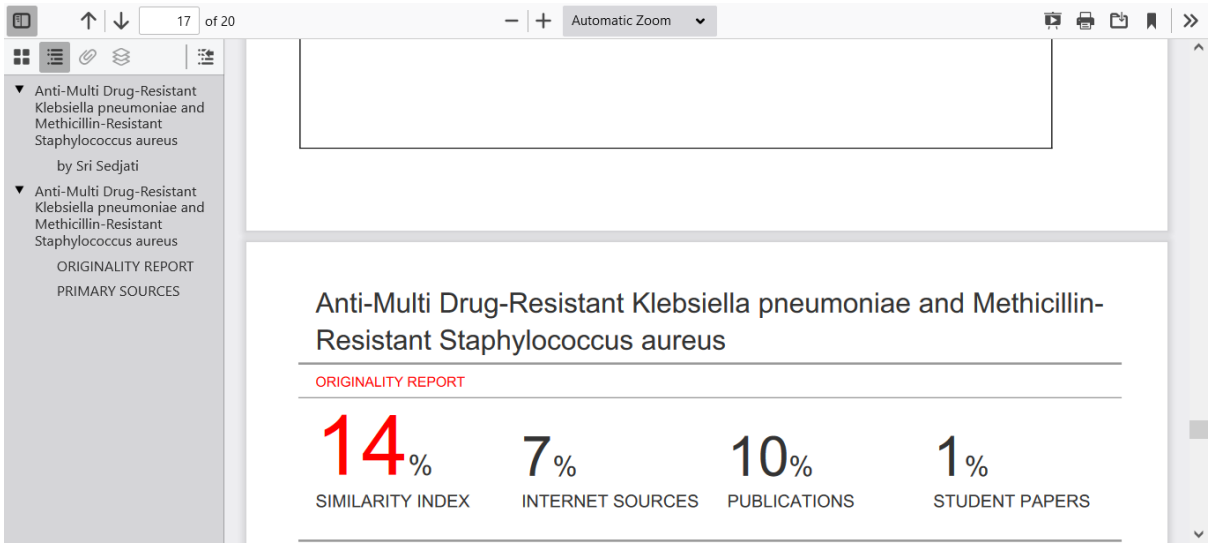

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Dear Dr. Sri Sedjati **February 19, 2021**

Anti-Multi Drug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibranchiatum* Extract

Manuscript Number: JJBS 326\20

Thank you for submitting the above mentioned manuscript to Jordan Journal of Biological Sciences (JJBS). It has been considered by an Editor, a Member of the Editorial Board and three independent expert referees. They agree that the paper presents an interesting piece of work, however, before your paper can be accepted for publication, several changes are required (**Including language editing as recommended by editorial office**); you should also respond satisfactorily to some questions of methodology and interpretation which have been raised by the referees (See attached and Comments Below). **The current decision does not guarantee an automatic accept decision following revision and the handling editor may still reject your manuscript. Please make sure that all References are according to JJBS Format as well as you send me the consent form and the results of plagiarism software (I authenticate or turnitin) with similarity results less than 15%.**

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Jordan Journal of Biological Sciences **Deanship of Scientific Research**

Manuscript Evaluation Report- Referee 1

Manuscript ID: JJBS 326/20 **Due date: January 18, 2021**

MS Title: Anti-Multi Drug-Resistant Klebsiella pneumoniae and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibranchiatum* Extract

Type of Article: ☐ Review Article ☐ Research Paper ☐ Case Report

PART A:

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NO.	Criteria	Score
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4	**What is the scholarly quality of the manuscript?**	4
5	**Is this a new and/ or original contribution?**	4
6	**Is the research methodology utilized appropriate and properly administered?**	5
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8	**Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?**	4
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6

PART B: Comments per Section of Manuscript:

Abstract	See attachment
Introduction	See attachment
Methodology	See attachment
Results	See attachment
Discussion and Conclusion	See attachment
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PART C: Recommendation (Kindly Mark With An ✓)

Acceptable in its Present Form	
Acceptable with Minor Revision	✓
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Manuscript Evaluation Report- Referee 2

Manuscript ID: JJBS 326/20 **Due date: February 18, 2021**

MS Title: Anti-Multi Drug-Resistant Klebsiella pneumoniae and Methicillin-Resistant *Staphylococcus aureus* from Active Fraction of Sponge-Association Fungus *Trichoderma longibranchiatum* Extract

Type of Article: ☐ Review Article ☐ Research Paper ☐ Case Report

PART A:

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5	**Is this a new and/ or original contribution?**	
6	**Is the research methodology utilized appropriate and properly administered?**	4
7	**Are the methods of data analysis acceptable?**	4
8	**Are the results and conclusions clear, adequately presented and organized in relation to rest of manuscript?**	4
9	**Are the illustrations and tables necessary and in acceptable format?**	4
10	**Are the interpretations/ conclusions sound and justified by the data?**	4
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Introduction	
Methodology	- Results describes in this manuscript are interesting, however it is not correctly written in English language. Thus English must be greatly improved in the whole document and particularly in the 'Materials and Methods' section. - Petri dishes should be written with a capital letter for the P of Petri.
Results	- Section 3.1. : 'can grow well in MEA and can be modified …' : what do you mean by 'modified' please be more precise. - Figures 1 and 3 : letters 'a' and 'b' are not well assigned to the figures
Discussion and Conclusion	- Do modifications in culture conditions of *Trichoderma longibranchiatum* influence/affect production of the described active fraction?
References	several references given in the text are not listed and *vice versa* for 2 references (Farhat et al., 2009 ; Marcellano et al., 2017) that are cited in the list but not mentioned in the text). -

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Acceptable with Minor Revision	✓
Reconsidered after Major Revision	
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Sri Sedjati
Department of Marine Science, Faculty of Fisheries and Marine Science,
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- Respons to Referee 1

Respons to Referee #1

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Abstract	See attachment--→I've revised it.
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Methodology	See attachment--→I've revised it.
Results	See attachment--→I've revised it.
Discussion and Conclusion	See attachment--→I've revised it.
References	See attachment--→I've revised it.

- Respons to Referee 2

Respons to Referee #2

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Abstract	
Introduction	
Methodology	- Results describes in this manuscript are interesting, however it is not correctly written in English language. Thus English must be greatly improved in the whole document and particularly in the 'Materials and Methods' section. I've revised it and tried to rewrite the materials and methods better and clearly. - Petri dishes should be written with a capital letter for the P of Petri. Yes, I've corrected it.
Results	- Section 3.1. : 'can grow well in MEA and can be modified …' : what do you mean by 'modified' please be more precise. The modified media is prepared by replacing malt extract with a cheaper material, namely fish and cassava extracts. All of them produce secondary metabolites that have antibacterial activities, but the best is obtained from ethyl acetate extract of fungus that are cultivated with MEA. - Figures 1 and 3 : letters 'a' and 'b' are not well assigned to the figures Yes, I've corrected it.
Discussion and Conclusion	- Do modifications in culture conditions of *Trichoderma longibranchiatum* influence/affect production of the described active fraction? Yes. It has been published in Sedjati *et al*. (2020). This type of media affects the production of secondary metabolites, but only secondary metabolites of MEA media are traced to their active fraction.
References	several references given in the text are not listed and *vice versa* for 2 references (Farhat et al., 2009 ; Marcellano et al., 2017) that are cited in the list but not mentioned in the text). - Yes, I've corrected it -

- Revised manuscript #1

Manuscript Number JJBS 326/20

Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus*

Sri Sedjati [1,4]*, Ambariyanto Ambariyanto[1,2], Agus Trianto [1,2], Ali Ridlo[1], Endang Supriyantini[1], Agus Sabdono [1,3], Ocky Karna Radjasa[1,3], Teguh Firmansyah[2]

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Abstract

Extracts from sponge-associated fungus *Trichoderma longibrachiatum* have been studied and contains antibacterial compounds which can inhibit several pathogenic multidrug-resistant organisms. This study aims to determine the active fraction of the extract which is antibacterial against the gram-negative Multi Drug-Resistant *Klebsiella*. *pneumoniae* pathogen and gram-positive Methicillin-Resistant *Staphylococcus aureus*. In this study, the fungus was cultivated using solid media of malt extract agar (MEA) for 6-9 days (24 hours dark, static, pH 5.6, 60 ‰ salinity, and 27 ºC). The mycelia and media were macerated by methanol and then partitioned using ethyl acetate. Active fraction tracing was carried out using the bioautography method and then isolated by the open column chromatography method. Antibacterial activity testing was done using the Broth Dilution method to determine the Minimum Inhibitory Concentration (MIC). The results of the study showed that ethyl acetate extract contained one active fraction (R_f value = 0.14), which has reactive characteristics on vanillin reagent and absorbed ultraviolet light (λ 375.5 nm absorbance peak). The active fraction was able to inhibit the growth of MDR *K. pneumoniae* and MRSA bacteria at the same MIC value, i.e. 256 µg mL^{-1}. In conclusion, an active fraction of *T. longibrachiatum* can be developed as an antibacterial against MDR *K. pneumoniae* and MRSA.

Keywords: Sponge-Associated Fungus, Active Fraction, Antibacterial, Minimum Inhibitory Concentration

1. Introduction

Klebsiella pneumoniae (*K. pneumoniae*) is an opportunistic pathogen, can be categorized in gram-negative group bacteria which has thick, non-motile, facultatively anaerobic, and rod-shaped cell walls. These bacteria produce Extended Spectrum β-Lactamases (ESBL) which can degrade certain antibiotics (β-lactam group), such as

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penicillin and cephalosporin, so they become inactive (Farhat *et al*., 2009). Also, it is protected by a capsule (composed of polysaccharides), both of which will further increase its pathogenicity. The infectious diseases caused by them are such as liver abscess, bacteremia, lung infection, acute leukemia, meningitis, and the bacteria may even cause death (Turton *et al*., 2010; Adwan *et al*., 2020)). Hospitalized patients with weak immunity are the main target for this bacterial attack. In current conditions, there is a tendency to increase the prevalence of infection caused by *K. pneumonia* along with the decrease in sensitivity to antibiotics used to treat the infection (Li *et al.*, 2014; Santana *et al*., 2016). According to Adwan *et al*. (2020), the prevalence of capsular polysaccharide genes among *K. pneumoniae* and high level of drug resistance will make bacterial infections are increasingly widespread, both in the hospital environment and community which leads to failure of treatment.

Staphylococcus aureus (*S. aureus*) is another cause of some dangerous infection. *S. aureus* is gram-positive, coccus-shaped, non-spore-forming, non-motile, facultative anaerobes, and forms a biofilm. In particular, biofilm formation by Methicillin-Resistant *Staphylococcus aureus* (MRSA) infection make difficult treatment and causes a hard prognosis (Sato *et al*., 2019). For a long time, the infection has been treated by such semi-synthetic penicillin antibiotics as methicillin, and it is considered the first representative of multidrug-resistant bacteria (MDR) since 1961s (Gajdács, 2019). MRSA incidence and prevalence are more likely to occur in health care/hospital settings area than in the community environment. These bacteria produce enterotoxins (exotoxins), ESBL enzymes, and immune modulators (Abbas *et al*., 2015).

According to Narendran and Kathiresan (2016) as well as Basiriya *et al.* (2017), some species of *Trichoderma* sp. have been screened and eventually have the ability to synthesis some antibacterial compounds. Moreover, some studies reported that ethyl acetate extract of *Trichoderma* sp. has antibacterial activity against pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Bacillus cereus*, and *S. aureus*,). These fungi isolated from the mangrove rhizosphere could be used as a producer of secondary metabolites to be developed into a new antibiotic against resistant bacteria. This was also stated by Synytsya *et al.* (2017) who investigated antibacterial compounds derived from ethanol and petroleum ether extracts. Some other researchers stated that the potential of antibacterial compounds produced by *Trichoderma* sp., such as *trichodin* A and B, pyridoxatin have antibacterial activity

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against *Staphylococcus epidermidis* and *S. aureus* (Wu *et al.*, 2014; Wang *et al.*, 2020), and trichodermaquinone to be antibacterial compound against MRSA (Khamthong *et al*., 2012). There are many classes of secondary metabolite considered as antibacterial compounds from marine fungi, such as glycopeptides, peptides, proteins, lipopeptides, aminolipopeptides, polyketides, polybrominated biphenyl ether, cyclic depsipeptides, terpenes, pentaketides, alkaloids, diketopiperazins, anthraquinones, chromones, steroids, lactones, quinolone derivatives, trisindole derivatives, macrolactam, and phenol derivatives (Thomas *et al*., 2010; Nalini *et al*., 2018; Wang *et al*., 2020).

A review by Li *et al.* (2019) showed that *Trichoderma* spp. can produce many metabolites with different bioactivities. These fungi are commonly distributed in many ecosystems, including the sea. The investigation by Sedjati *et al.* (2020) proved that ethyl acetate extract of sponge-association fungus *T. longibrachiatum* contains compounds that have antibacterial activity against MRSA and *K. pneumoniae*. Based on these findings, this study aims to determine the active fraction in ethyl acetate extract of *T. longibrachiatum* using autobiographical methods by the guidelines of its bioactivity test results.

2. Material and Methods

2.1. Fungus Isolate

The sample used in the study was from the sponge-associated fungus obtained from Falajava Beach, Ternate Island, North Maluku, Indonesia (00°47'09.12" N; 127°23'21.76" E coordinates) with TE-PF-03.1 code. The fungi have been identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence, and confirmed as *T. longibrachiatum* macro and microscopically (Sedjati *et al*., 2020).

2.2. Bacterial Pathogen

The test bacteria used in this experimental study were MDR *K. pneumoniae* obtained from Microbiology Laboratory, Diponegoro National Hospital, and MRSA from the University of Indonesia. Pathogenic bacteria were recultured being used for antibacterial testing. The process was done by taking bacterial stock colonies and transferring them into Mueller-Hinton Broth (MHB; Oxoid) and further incubated at 37°C for 24 hours.

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Commented [u13]: What do you mean by ITS?

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2.3. Fungus Cultivation

Fungus cultivation according to the method by Sedjati *et al*. (2020). *T. longibrachiatum* isolate coded TE-PF-03.1 was subcultured using Malt Extract Agar (MEA; Merck). Then, the mycelia were were taken about 2 mm in diameter and cultivated on new MEA media. The treatment of cultivation periods was carried out at 6,7,8, and 9 days (static, 24 hours in dark, pH 5.6, salinity 60‰, temperature 27^{o}C). MEA preparation was conducted by using sterile seawater (solid, 20 mL media/Petri dish).

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2.4. Extraction and Determining Extract Weight

After the cultivation period has finished, the media and the mycelia were cut into small pieces and then macerated with methanol (1:1v/v), filtered using Whatman paper no. 42 and the filtrate were evaporated at a rotary evaporator with 40^{o}C and low pressure. Furthermore, partitioning of the fungal extract was done using methanol-distilled water (50%) and ethyl acetate (1:1v/v). Moreover, each fraction was evaporated using rota vapor to be methanol and ethyl acetate extracts and then these were weighed.

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Is it a standard method? If it, add reference please

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2.5. Profiling of Secondary Metabolites

The Thin Layer Chromatography (TLC) method (Harborne, 1984) was used for profiling chemical extracts. There were in total 10 µl of extract solution in methanol (1 mg/ml) was spotted on the baseline of the TLC plate (Merck, silica gel 60 F254). Several mobile phases (n-hexane, ethyl acetate, chloroform, acetone, and methanol) with various comparisons were experimented with to elute the extracts until obtaining a completely separated spot profile. The mobile phases used were sequential based on polarity levels, i.e. a mixture of n-hexane and ethyl acetate (4:1; 3:2; 2:3; 1:4, and 0:5). Spot identification using the value of R_f (Retention Factor) and spraying with staining reagents. After the elution process, TLC was visualized by UV light (365 nm), 2% vanillin-H_2SO_4, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (Harborne, 1984; Sen *et al*., 2012; Trianto *et al*., 2019). Furthermore, the TLC plate was heated at 110 ^{o}C for 2-3 minutes. The same method was used to detect the active antibacterial fraction after the isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

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Add them please

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2.6. Antibacterial Activity Test

The antibacterial activity test using a disc diffusion assay method to determine the inhibition zone against pathogen growth. Pathogenic bacteria were cultured on Mueller-Hinton Agar (MHA; Oxoid) with an initial density equivalent to 0.5 McFarland (1.5 x10^8 CFU mL^{-1}). Extract in the dimethylsulfoxide (DMSO) solvent was tested against pathogenic bacteria. 10 µL of extract solution was dropped onto the sterile disc paper (6 mm diameter; Oxoid) with 500 µg/disc concentration. The negative control used DMSO and the positive control used chloramphenicol (30µg/disc; Oxoid). The inhibition zone was measured after 24 hours of incubation at 37 ºC (Trianto *et al*., 2017).

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2.5. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured on MHA media with 1.5 x10^8 CFU mL^{-1} initial density. The extract in ethyl acetate solvent (10 µL, 10 mg mL^{-1} concentration) was spotted on the TLC surface baseline and eluted with a suitable mobile phase to produce perfectly separated spots. TLC plate was applied with silica surface attached to the MHA media surface (facing downward) and left for 60 minutes. Furthermore, the TLC plate was removed from the test bacteria medium and the Petri dishes were closed. All processes were carried out in a laminar flow cabinet. The incubation was carried out for 24 hours at 37 ºC and the formation of the inhibition zone around the TLC spot was carefully observed. The spots around which the next inhibition zone appears were called an active fraction.

2.6. Active Fraction Isolation

The active fractions found were isolated using the open column chromatography (OCC) method with the appropriate mobile phase (referring to the TLC profile). The column was filled with silica gel (60–120 mesh) mixed with n-hexane: ethyl acetate (1: 1) solvent. The extract was dissolved in the solvent and was slowly being loaded on the top surface of the silica gel. The extract was then eluted using a solvent sequence based on the polarity increase. Furthermore, the eluate coming out was collected using a test tube (every 10 mL of eluate), checked again using the TLC method, and the same eluates were put together. The eluates containing active fraction were concentrated at a rotary evaporator for further testing.

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Commented [SS28R27]: It is standard method.

2.7. Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was conducted based on the Broth Dilution method. MIC determination refers to the method proposed by Sowjanya *et al.* (2015) and Fajarningsih *et al.* (2018) using 96-well microplates with resazurin (Sigma-Aldrich) as an indicator of the viability of the test bacteria (REMA assay). A total of 100 µL of the extract solution in the DMSO solvent with the highest concentration (2,048 µg mL^{-1}) was filled in the first well in certain rows. The next well was filled with 50 µL of sterile MHB nutrients. The 50 µL test material was transferred from the first well to the next well to reach serial dilution (at wells no. 1-10). Then, 30 µl of resazurin solution (0.02% in distilled water) was added to each well. At last, 10 µl of the bacterial suspension (1.5 $x10^8$ CFU mL^{-1}) was added to each well. Chloramphenicol was used as a positive control (the highest concentration was at 64 µg mL^{-1}) and DMSO as a negative control (at well no. 11). The well contained MHB without extract as growth media control (at well no. 12). The microplate was incubated at 37 ºC for 24 hours. After the incubation period, the well functioning as growth control would appear pink. The MIC value was determined based on the lowest concentration which could inhibit the growth of the tested bacteria.

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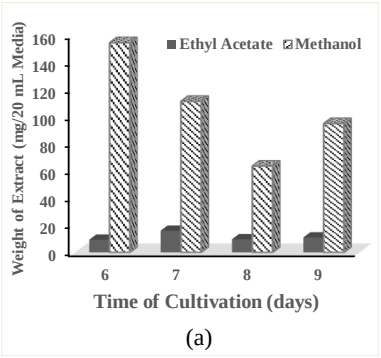
3. Results and Discussion

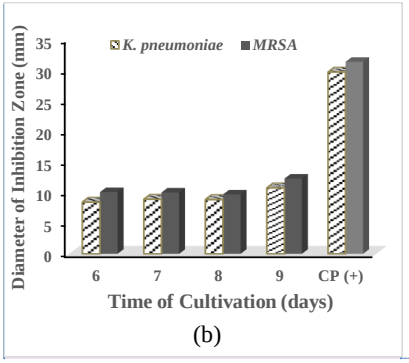
3.1. Assessment of Antibacterial Potential

T. longibrachiatum species is one of the fungi species which are easy to cultivate. It can grow well in MEA and modified media. The modified media is prepared by replacing malt extract with a cheaper material, namely fish and cassava extracts. All of them produce secondary metabolites that have antibacterial activities, but the best is obtained from ethyl acetate extract of fungus cultivated with MEA. The best potential reached is its antibacterial properties against *K. pneumoniae* and MRSA (Sedjati *et al*., 2020). Data from the results of this study indicated that the secondary metabolite product of *T. longibrachiatum* cultivated in MEA was mostly in the form of methanol extract (polar compound) and only a small part of them was ethyl acetate extract (semi-polar to non-polar compound). The weight of fungal extract is based on the polarity of its secondary metabolites after 6-9 days cultivation periods described in (Figure 1a). Ethyl acetate extract was produced in only small amounts. On the other hand, the ethyl acetate extract has antibacterial activity against

these two pathogens as shown in (Figure 1b). The greatest potential as an antibacterial was seen in the ethyl acetate extract from the fungus which had been cultivated for 9 days.



(a)



(b)

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Is it Chloramphenicol?
If it is, explain it please.

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Figure 1. Characteristics of *T. longibrachiatum Extract*: (a) Extract Weight on Polarity basis; (b) Antibacterial Potential of Ethyl Acetate Extract Against MDR Pathogens *K. pneumoniae* and MRSA (note: CP= Chloramphenicol)

Some synthesized fungal secondary metabolites are only in small amounts because they are not for the main energy supply needed by the fungus and are only made at suboptimal conditions as a response to environmental pressure (Nielsen and Nielsen, 2017). The peak of secondary metabolite production in this study occurs when the fungal life cycle was in a stationary phase. This statement conforms to several research results stating that the fungus has entered a stationary period on day 6 to 9 after being cultivated (Gliseida *et al.*, 2013; Arumugam *et al.*, 2015). Methanol extract seemed to predominate over the extract of *T. longibraciatum.* However, when tested for antagonists against *K. pneumoniae* and MRSA at a concentration of 500 µg/disc, they did not show antibacterial activity. This fact is similar to the research result of Leylaie and Zafari (2018). In general, the ethyl acetate extract metabolite of the *T. longibrachiatum* is more likely to be antibacterial than its methanol extract. According to the statement of Chamekh *et al.* (2019), methanol extract is presumed to contain enzymes synthesized by *T. longibrachiatum* for external digestion, along with several units of saccharides, amines/peptides, fatty acids/glycerol which are hydrolysis results of organic compounds in the media. Polar metabolites dissolved in

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methanol consists of enzymes (such as amylase, protease, and lipase) which are synthesized by fungi to degrade the nutrients in the media. Based on the research of Massadeh *et al*. (2010), the fungi can utilize a variety of carbon sources and produce various ligninolytic and cellulolytic enzymes. Added by Muthulakshmi *et al*. (2011), protease is produced by fungi from the first day of cultivation and reaches its peak on the 7th day (wheat bran as a media, pH 5.0, temperature 30^{0}C)

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3.2. Secondary Metabolite of Fungal Extract

Chemical compounds contained in ethyl acetate extract of *T. longibraciatum* which was cultivated for 9 days can be traced based on its TLC profile as shown in Figure 2. Only ethyl acetate extract was used for the next stage of research since methanol extract is not potentially antibacterial. The best spot separation was seen in the results of TLC with a mobile phase with a 2:3 ratio as seen in Figure 2 (3rd order). Based on the number of spots that appeared, at least 5 compounds were detected with R_f values: 0.14, 0.26, 0.57, 0.71, and 0.89. As congenial with the order of R_f values, the compound with the smallest R_f value is relatively the most polar, while the largest R_f is the most non-polar.

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Commented [SS37R36]: Methanol extract does not produce a inhibition zone.

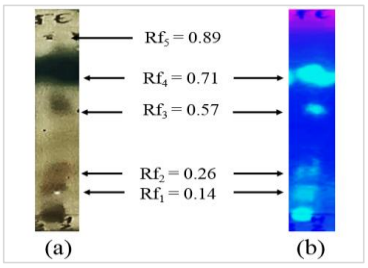

Figure 2. TLC Profile of Ethyl Acetate Extract Using Mobile Phase of n-Hexane and Ethyl Acetate (2:3) : (a) Visualization Results with 365 nm UV Light; (b) Visualization Results with Vanillin-H2SO4 1%

Compound prediction in ethyl acetate extract of *T. longibrachiatum* was traced based on previous studies' references. The spot looks fluorescent blue when exposed to UV indicating that the organic compound has a double bond (polyene or conjugated compound). The increased wavelength of the UVs (200-400 nm) absorbed indicates that the number of double bonds also increased (Hamilton-Miller, 1973; Mohammed, 2018). Besides, compounds reacting positively with the vanillin indicates the presence of carbonyl functional groups that contain a carbon-oxygen double bond (aldehydes,

ketones). Accordingly, these compounds probably are from terpenoids, fatty acids/essential oils, steroids, flavonoids, or phenolic groups. A compound that reacts negatively to ninhydrin shows that it is not a nitrogen compound or its derivative. In contrast, negative to ferric (III) chloride indicates that the compound does not have a phenol functional group (Harborne, 1984; Jork, 1990). In this study, several spots in the TLC profile of ethyl acetate extract reacted positively to 365 nm UV light and vanillin reagent, but all of them reacted negatively to ninhydrin and ferric (III) chloride (as shown in Figure 2).

3.3. Active Fraction as Antibacterial Against *K. pneumoniae* and MRSA

After an autobiography test was conducted on *K. pneumoniae* and MRSA pathogens, it was found that the spot with the smallest R_f (0.14) was the active fraction as antibacterial. The results of the autobiography test will help detect the presence of antibacterial compounds by the formation of an inhibition zone around the active spot as shown in Figure 3.

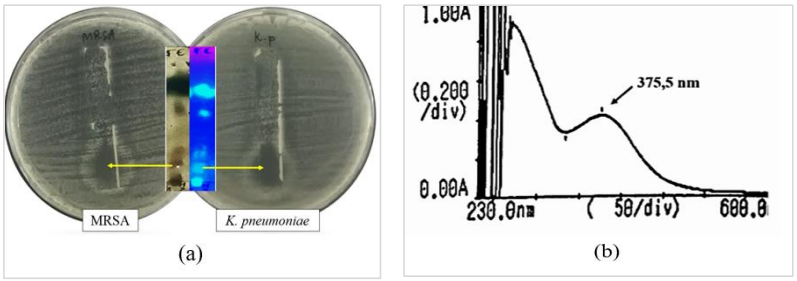

Figure 3. Determination of Active Fraction as Antibacterial **:** (a) Autobiographical Results of Ethyl Acetate Extract Against MRSA and MDR *K. pneumoniae*, (b) Characteristics of Antibacterial Active Fraction Based on Spectra Patterns towards UV Light Absorbance

The active fraction was reactive to vanillin and 365 nm UV light on TLC visualization results and was strengthened by the active fraction spectra pattern using a UV-Vis spectrophotometer which has λ 375.5 nm absorption peak (illustrated in Figure 3b). Based on the description of this characteristic, the active fraction is likely thought to have a carbonyl group and contain conjugated double bonds.

3.4. Minimum Inhibitory Concentration of Active Fraction

Antibacterial activity of the active fraction against MDR *K. pneumoniae* and MRSA pathogens resulted in a similar MIC value, i.e. at 256 μg mL^{-1}. *K. pneumoniae* bacteria are categorized as gram-negative bacteria, while MRSA is gram-positive. Both bacteria are still sensitive to chloramphenicol antibacterial since their MIC value is less than 8 μg mL^{-1}.

The cell wall of gram-negative bacteria is thinner, composed of peptidoglycan, 2 layers of phospholipids, and is protected by a lipopolysaccharide capsule. Gram-positive bacteria have thicker walls composed of peptidoglycan and lipoteichoic acid, and 1 layer of phospholipids (Lima *et al*., 2013). Both bacteria are still sensitive to chloramphenicol antibacterial since their MIC value is less than 8 μg mL^{-1} (CLSI, 2017). Chloramphenicol is a commercial broad-spectrum antibacterial. Moreover, chloramphenicol can damage important metabolic pathogens by binding the 50S ribosome subunit and blocking essential ribosomal function. The interaction of the nitrobenzyl functional group from chloramphenicol and the bacterial RNA nitrogen base may interfere with the formation of peptides during the process of protein biosynthesis done by bacteria (Kostopoulou *et al*., 2011).

Commented [u38]: Why you choose chloramphenicol? Although its highly toxic and it does not used in treatment nowadays except in certain cases.

Commented [SS39R38]: •Chloramphenicol only as a standard comparison of antibacterial activity
•Chloramphenicol is an antibiotic that is effective against gram-positive and negative bacteria
•Test bacteria used have not been resistant to chloramphenicol

The active fraction resulted from this study had a carbonyl group and also alternating double bonds (conjugation). The aldehyde and ketone carbonyl groups are highly polarized because carbon is less electronegative than oxygen. Carbon contains a partial positive charge (δ^+), while oxygen has a partial negative charge (δ^-). Hence, the carbonyl group can function as a nucleophile and an electrophile. The conjugation of a double bond to the carbonyl group will transmit the electrophilic character of the carbonyl to the beta-carbon of the other double bonds, or popularly called charge delocalization (Sarker and Nahar, 2007). Charged compounds ions will make them easier to interact with bacterial cell wall so that they can penetrate the cytoplasm membrane.

The mechanism of action of the active fraction as an antibacterial is assumed to be related to its ability to form electrophile sites, i.e. C with δ^+ partial charge which will electrostatically interact with the phospholipid head (PO_4^-) on the surface of the bacterial cell wall. According to Malanovic and Lohner (2016), a positive charge is essential for the initial binding to the surface of the bacterial membrane with a negative charge, which allows it to enter the bacterial cell membrane. Furthermore,

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these active compounds can affect the metabolic activity of bacterial cells which will eventually cause growth retardation or even death of pathogens.

T. longibrachiatum fungus does not only live in association with sponges in the sea. However, it has also been previously found in soft corals from the water of Panjang Island, Central Java. In addition, its ethyl acetate extract was able to inhibit the growth of MDR-*S. haemolyticus* and produced a 12.2 mm inhibition zone at a concentration of 300 µg/disc (Sabdaningsih *et al*., 2017). The secondary metabolite from the same fungus has been published by Sperry *et al.* (1998). The ethyl acetate extract of *T. longibrachiatum* is associated with *Haliclona sp*. sponge from Sulawesi water containing an epoxysorbicillinol ($C_{14}H_{16}O_5$), is a member of sorbicillinoids (vertinoids) polyketide compounds. According to Harned and Volp (2011); Meng *et al*. (2016); Salo *et al*. (2016), sorbillinoids are secondary metabolites of hexaketide that undergo cyclization at the carboxylate terminus. Its chemical structure has several double bonds and carbonyl groups. The results of a study from Corral *et al*. (2018) showed that some of these have antibacterial activity, such as sorbicillin ($C_{14}H_{16}O_3$), sorbicillinol ($C_{14}H_{16}O_4$), dihydrosorbicillin ($C_{14}H_{18}O_4$), oxosorbicillinol ($C_{14}H_{16}O_5$), bisvertinol ($C_{28}H_{34}O_8$), and bisvertinolone ($C_{28}H_{32}O_9$). These compounds can inhibit pathogens *Acinetobacter baumannii*, *P. aeruginosa*, *S. aureus*, and *K. pneumoniae*.

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What do you mean?

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4. Conclusion

T. longibrachiatum fungi extracts contain an active fraction that can be developed as an antibacterial against gram-negative pathogens *MDR K. pneumoniae* and gram-positive MRSA. The active fraction is assumed to contain a carbonyl functional group and a conjugated double bond. The mechanism of its antibacterial action is related to the formation of electrophile sites on carbon. Thus, electrostatic interactions occur with negative charges on the cell walls of both gram-positive and gram-negative bacteria making it possible to penetrate the cytoplasmic wall. The active fraction of ethyl acetate extract was antibacterial against pathogens MDR *K. pneumoniae* and MRSA with the same MIC value, i.e. 256 µg mL^{-1}.

Commented [u46]: I think the value of 256 µg mL^{-1} is high in comparison with used antibiotic

Commented [SS47R46]: Yes, because my extract is still an active fraction, not a pure compound.

Acknowledgment

This scientific paper was written based on a research study supported by the Grant Program of Faculty of Fisheries and Marine Science, Diponegoro University. The funding was intended for research activities from the Fiscal year 2020 with contract number: 026/UN 7.5.10.2/PP/2020.

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Table 1. MIC Value of Active Fraction and Chloramphenicol Against MDR *K. pneumoniae* and MRSA Pathogens

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Please read instructions carefully

Test Material	Value of MIC (μg mL^{-1}) against Pathogens	
	MRSA	*K. pneumoniae*
Active Fraction	256	256
Chloramphenicol	4*	4*

*MIC>32 = resistant, 18-32 =intermediate, <8 =sensitive (CLSI, 2017)

6. Decision by Reviewers

- Email: Both reviewers agree revised Manuscript ID: JJBS 326/20 & references correction

Jordan Journal Biological <jjbs@hu.edu.jo> Mar 11, 2021, 1:12 PM
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Dear Dr. Sri,
Good day. Both reviewers were satisfied with the revised manuscript. However, before I send you the acceptance letter, please revise the list of references and make sure it is written according to JJBS style. Thank you

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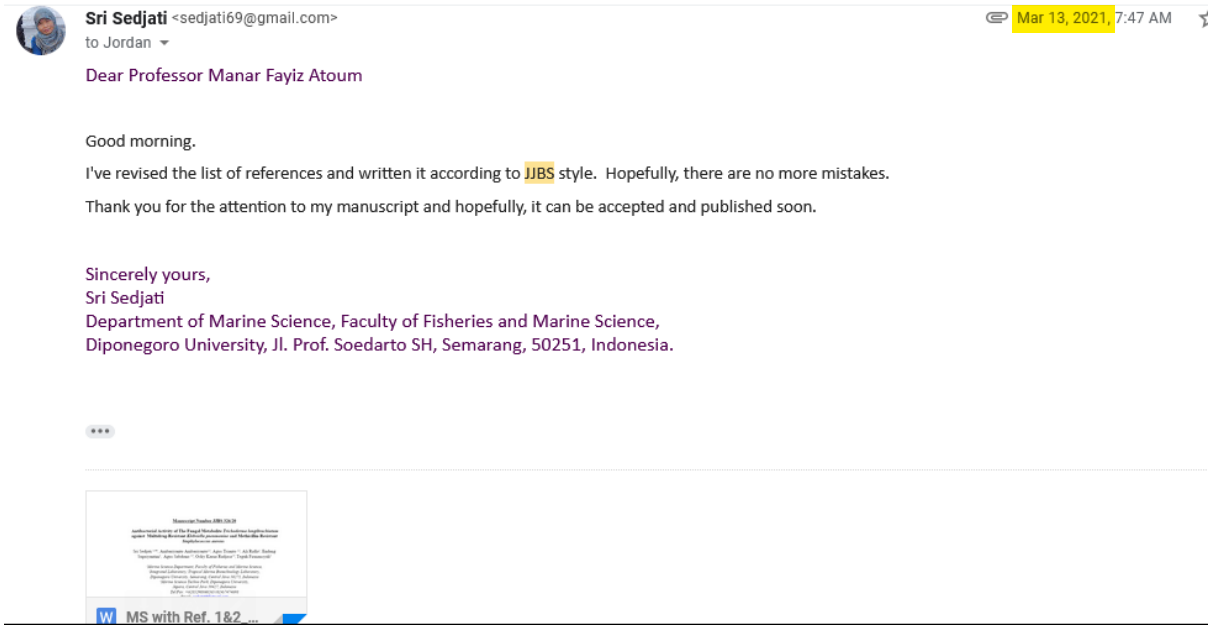
7. Submit references correction

- Email: references in JJBS style



Sri Sedjati <sedjati69@gmail.com> Mar 13, 2021, 7:47 AM
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Dear Professor Manar Fayiz Atoum

Good morning.

I've revised the list of references and written it according to JJBS style. Hopefully, there are no more mistakes.

Thank you for the attention to my manuscript and hopefully, it can be accepted and published soon.

Sincerely yours,
Sri Sedjati
Department of Marine Science, Faculty of Fisheries and Marine Science,
Diponegoro University, Jl. Prof. Soedarto SH, Semarang, 50251, Indonesia.

MS with Ref. 1&2_...

- Corrected References

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8. Decision and Acceptance

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Dear Dr. Sri Sedjati **March 15, 2021**

Manuscript Title: **Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus***

Sri Sedjati [1,4*], Ambariyanto Ambariyanto[1,2], Agus Trianto [1,2], Ali Ridlo[1], Endang Supriyantini[1], Agus Sabdono [1,3], Ocky Karna Radjasa[1,3], Teguh Firmansyah[2]

[1]*Marine Science Department, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang, Central Java 50275, Indonesia* [2]*Integrated Laboratory,* [3]*Tropical Marine Biotechnology Laboratory,* [4]*Marine Science Techno Park, Diponegoro University, Jepara, Central Java 59427, Indonesia*
Tel/Fax: +6285290360265/(024)7474698

Received: December 10, 2020; Revised: March 6, 2021; Accepted: March 15, 2021

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- Corrections text

compounds that have antibacterial activity against MRSA and *K. pneumoniae*. Based on these findings, this study aims to determine the active fraction in ethyl acetate extract of *T. longibrachiatum* using bioautographical methods by the guidelines of its bioactivity test results.

2. Materials and Methods

2.1. Fungus Isolate

The sample used in the study was from the sponge-associated fungus obtained from Falajava Beach, Ternate Island, North Maluku, Indonesia (00°47'09.12" N; 127°23'21.76" E coordinates) with TE-PF-03.1 code. The fungi have been identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence, and confirmed as *T. longibrachiatum* macro and microscopically (Sedjati *et al*., 2020).

2.2. Bacterial Pathogen

The test bacteria used in this experimental study were MDR *K. pneumoniae* obtained from Microbiology Laboratory, Diponegoro National Hospital, and MRSA from the University of Indonesia. Pathogenic bacteria were recultured being used for antibacterial testing. The process was done by taking bacterial stock colonies and transferring them into Mueller Hinton Broth (MHB;

Trianto *et al*., 2019). Furthermore, the TLC plate was heated at 110 °C for 2-3 minutes. The same method was used to detect the active antibacterial fraction after the isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

2.6. Antibacterial Activity Test

The antibacterial activity test using a disc diffusion assay method to determine the inhibition zone against pathogen growth. Pathogenic bacteria were cultured on Mueller-Hinton Agar (MHA, Oxoid) with an initial density equivalent to 0.5 McFarland (1.5 x10^8 CFU mL^{-1}). Extract in the dimethylsulfoxide (DMSO) solvent was tested against pathogenic bacteria. 10 µL of extract solution was dropped onto the sterile disc paper (6 mm diameter; Oxoid) with 500 µg $disc^{-1}$ concentration. The negative control used DMSO and the positive control used chloramphenicol (30µg $disc^{-1}$; Oxoid). The inhibition zone was measured after 24 hours of incubation at 37 °C (Trianto *et al*., 2017).

2.7. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured on MHA media with 1.5 x10^8 CFU

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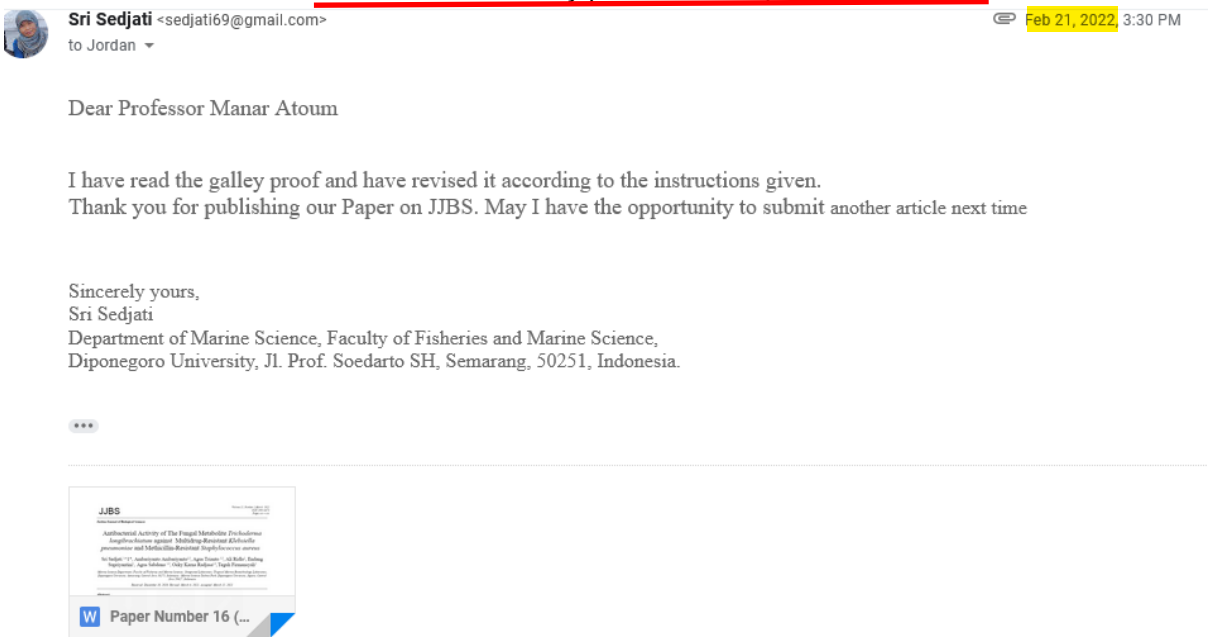

Sri Sedjati <sedjati69@gmail.com> Feb 21, 2022, 3:30 PM
to Jordan

Dear Professor Manar Atoum

I have read the galley proof and have revised it according to the instructions given.
Thank you for publishing our Paper on JJBS. May I have the opportunity to submit another article next time

Sincerely yours,
Sri Sedjati
Department of Marine Science, Faculty of Fisheries and Marine Science,
Diponegoro University, Jl. Prof. Soedarto SH, Semarang, 50251, Indonesia.

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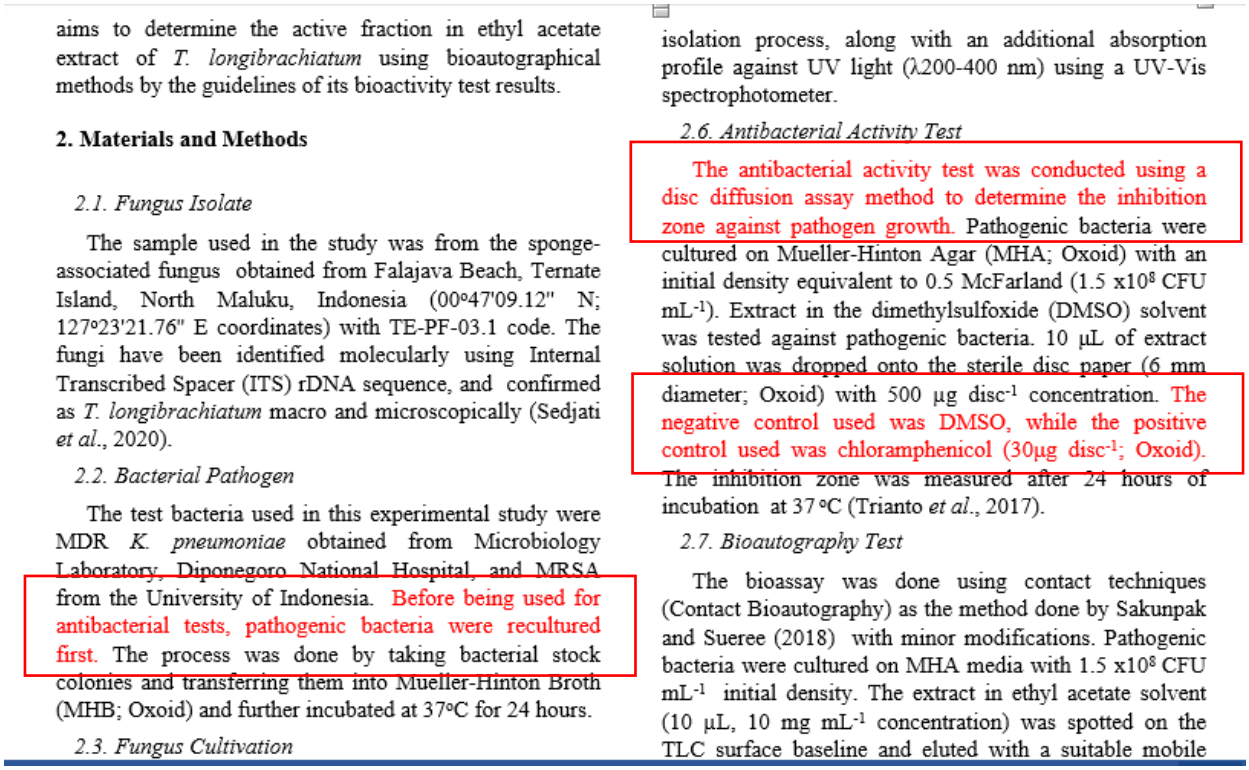
- Revisions text



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2.3. Fungus Cultivation

isolation process, along with an additional absorption profile against UV light (λ200-400 nm) using a UV-Vis spectrophotometer.

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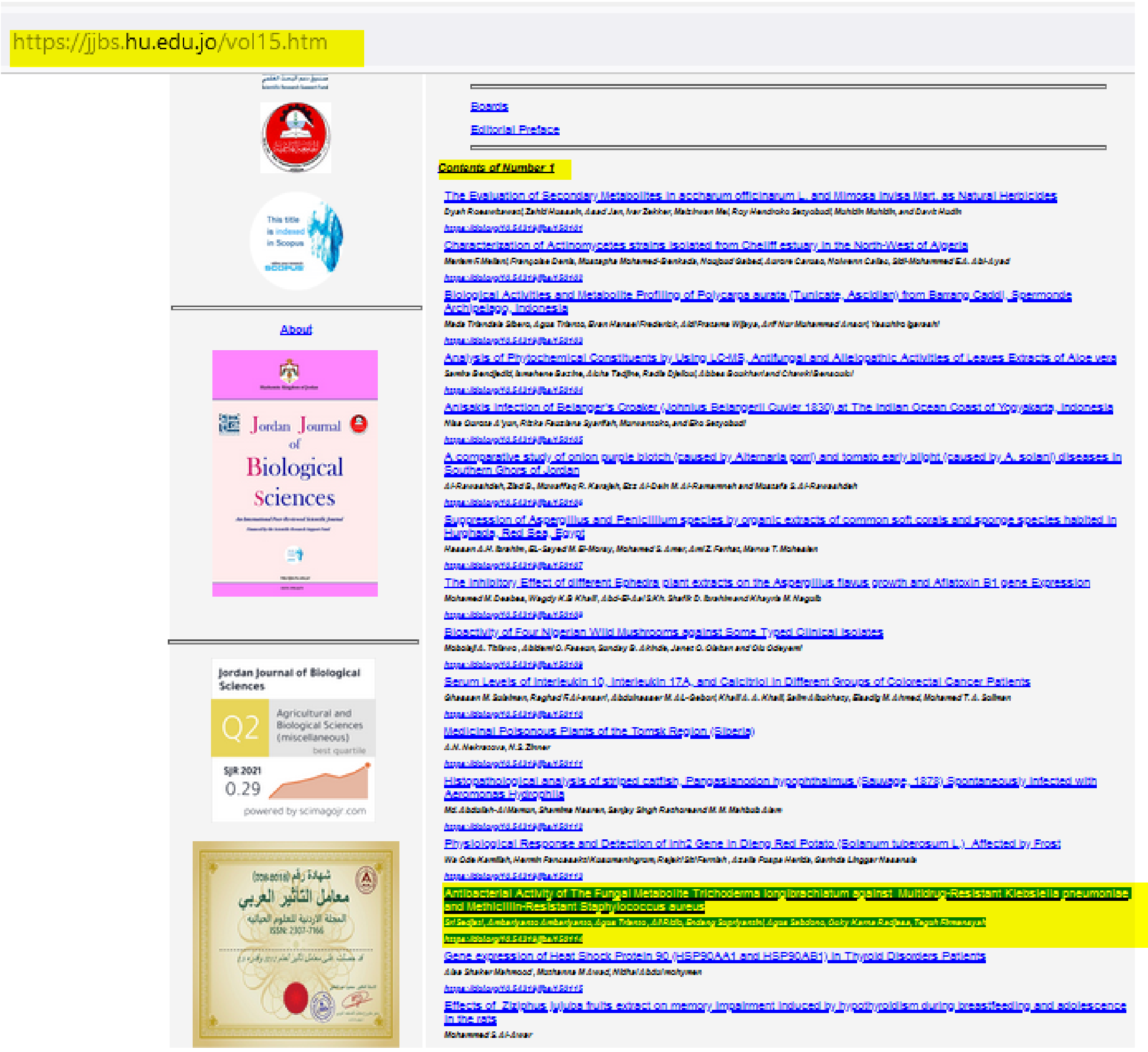
2.7. Bioautography Test

The bioassay was done using contact techniques (Contact Bioautography) as the method done by Sakunpak and Sueree (2018) with minor modifications. Pathogenic bacteria were cultured on MHA media with 1.5 x10^8 CFU mL^{-1} initial density. The extract in ethyl acetate solvent (10 µL, 10 mg mL^{-1} concentration) was spotted on the TLC surface baseline and eluted with a suitable mobile

11. Publish Online



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Antibacterial Activity of The Fungal Metabolite *Trichoderma longibrachiatum* against Multidrug-Resistant *Klebsiella pneumoniae* and Methicillin-Resistant *Staphylococcus aureus*

Sri Sedjati [1,4,*], Ambariyanto Ambariyanto[1,2], Agus Trianto [1,2], Ali Ridlo[1], Endang Supriyantini[1], Agus Sabdono [1,3], Ocky Karna Radjasa[1,3], Teguh Firmansyah[2]

[1]Marine Science Department, Faculty of Fisheries and Marine Science; [2]Integrated Laboratory, [3]Tropical Marine Biotechnology Laboratory, Diponegoro University, Semarang, Central Java 50275, Indonesia; [4]Marine Science Techno Park, Diponegoro University, Jepara, Central Java 59427, Indonesia

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Abstract

Extracts from sponge-associated fungus *Trichoderma longibrachiatum* have been studied and contain antibacterial compounds which can inhibit several pathogenic multidrug-resistant organisms. This study aims to determine the active fraction of the extract which is antibacterial against the gram-negative Multi Drug-Resistant *Klebsiella. pneumoniae* pathogen and gram-positive Methicillin-Resistant *Staphylococcus aureus*. In this study, the fungus was cultivated using solid media of malt extract agar (MEA) for 6-9 days (24 hours dark, static, pH 5.6, 60 ‰ salinity, and 27 °C). The mycelia and media were macerated by methanol and then partitioned using ethyl acetate. Active fraction tracing was carried out using the bioautography method and then isolated by the open column chromatography method. Antibacterial activity testing was done using the Broth Dilution method to determine the Minimum Inhibitory Concentration (MIC). The results of the study showed that ethyl acetate extract contained one active fraction (R_f value = 0.14), which has reactive characteristics on vanillin reagent and absorbed ultraviolet light (λ 375.5 nm absorbance peak). The active fraction was able to inhibit the growth of MDR *K. pneumoniae* and MRSA bacteria at the same MIC value, i.e. 256 µg mL^{-1}. In conclusion, an active fraction of *T. longibrachiatum* can be developed as an antibacterial against MDR *K. pneumoniae* and MRSA.

Keywords: Sponge-Associated Fungus, Active Fraction, Antibacterial, Minimum Inhibitory Concentration