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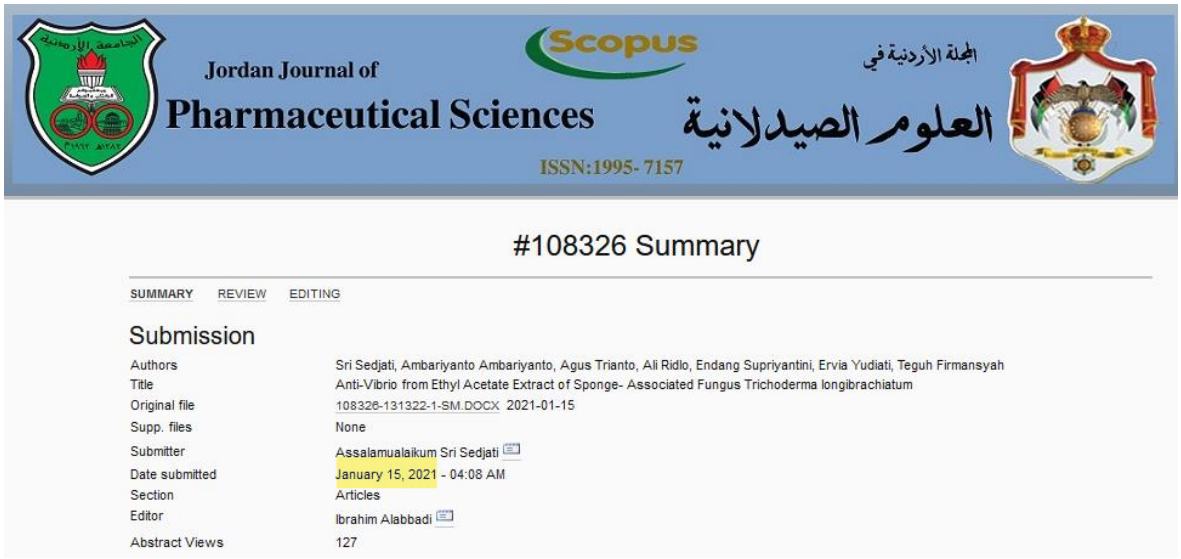

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Anti-Vibrio from Ethyl Acetate Extract of Sponge Associated Fungus *Trichoderma longibranchiatum*

ABSTRACT

Some of *Vibrio* spp. bacteria are pathogenic to humans and other organisms, including cultured fish or shrimp. This study aimed to determine the activity of ethyl acetate extract of *T. longibranchiatum* as an anti-vibrio fungus. The test bacteria used were: *V. harveyi, V. anguillarum, V. vulvinificus,* and *V. parahaemolyticus*. The fungus was cultured using Malt Extract Agar (MEA) medium for 9 days at 27^{o}C (static conditions, 24 hours dark, pH 5.6, and 60‰ salinity). Extracts obtained by maceration using ethyl acetate, followed by partitions using methanol (50%). Each fraction was concentrated to obtain polar ethyl acetate (PE) and semipolar ethyl acetate (SPE) extracts. The components of the constituent extract were traced with a Thin Layer Chromatography (TLC) and followed by ultraviolet spectrophotometry. The anti-vibrio activity was determined based on the value of Minimum Inhibitory Concentration (MIC). The results of the study showed that SPE was more potential to be used as anti-vibrio. The strongest activity was able to inhibit the growth of *V. vulvinificus* with 256 μg mL^{-1} MIC value while the weakest was against *V. parahaemolyticus* with 1.024 μg mL^{-1} MIC value. In conclusion, SPE has the potential to be developed as an anti-vibrio compound, particularly against *V. vulvinificus*.

Keywords: Ethyl Acetate Extract, Semipolar, Minimum Inhibitory Concentration

INTRODUCTION

Vibrio is a genus of bacteria found in a variety of freshwater and marine habitats. There are more than 100 species of *Vibrio* spp., and 12 of which cause infection in humans. *Vibrio cholerae* (*V. cholerae*) can cause cholera, a severe diarrheal disease that can be transmitted through contaminated water. Non-cholera *Vibrio* spp., such as *V. parahaemolyticus, V. alginolyticus,* and *V. vulnificus,* may cause vibriosis, an infection with various clinical expressions, and the mildest of which is gastroenteritis. Vibriosis infection is usually caused by exposure to seawater or by consuming contaminated raw or undercooked seafood. There is an exception, *V. vulnificus* which is an opportunistic pathogen causing wound infection which can rapidly lead to septicemia (1, 2). Infections caused by *V. vulnificus* are generally fatal. Therefore, accurate diagnosis and direct treatment are very important since the infection may cause death to the sufferer (3). antibiotics are widely used to treat vibriosis. The commonly used antibiotics include doxycycline, quinolone, cephalosporin, ciprofloxacin, tigecycline, and ceftazidime (2, 3). *Vibrio* spp. is a gram-negative bacterium that causes vibriosis in humans and animals. Moreover, fishery products and post-harvest products are common intermediaries for the transfer of bacteria from their original habitat to their new hosts.

The fisheries sector is one of the most fundamental fields for a country's food security. On the other hand, fisheries systems also play a major role in spreading vibriosis to humans. Vibriosis is a bacterial disease reported in Indonesian marine fish culture since the 1990s. The disease is reported mostly found in grouper and shrimp (monodon and vanname) culture, although infection also occurs in snapper (*Lates calcarifer*) and abalone (*Haliotis squamata*) aquaculture. The agent causing vibriosis in marine fish in Indonesia involves several vibrio species, including *V. harveyi, V. anguillarum, V. alginoluticus, V. parahaemolyticus* (4). Some

efforts are done to control vibriosis in fish farming activities still rely on the use of drugs or antibiotics given through oral and immersion. However, the leftover feed will eventually cause antibiotic contamination pollution in the water. Nowadays, several Vibrio species are even resistant to certain types of antibiotics. Based on the research (5) conducted on the North Coast of Java found that *V. parahaemolyticus* contaminating vanname shrimp is resistant to erythromycin (90%), amoxicillin-clavulanic acid (83.33%), and nitrofurantoin (58.33%).

Vibrio spp. live in marine habitats along with other bacterial species and microorganisms, like fungi. Competition among the inhabitants of an ecosystem will occur to compete for space and nutrition. Consequently, there is a possibility that there are any antagonistic species, both within among themselves and towards other species. Some research resulted in the fact that several marine-derived fungi species synthesize secondary metabolites of anti-vibrio as their chemical weapons to compete and to avoid predation. Several anti-vibrio metabolites found are such as ethyl acetate extract from sponge-associated fungus *T. asperellum* (6); prenylxanthone and aspergixanthones from the marine-derived fungus *Aspergillus sp*. cultured with shaken Czapek-Dox media (7); indole-diterpenoids and steroids isolated from *Penicillium janthinellum* (8), secondary metabolites of the mangrove-associated fungus *Aspergillus sp*. (9), and secondary metabolites of marine invertebrates associated fungi *A. flavus, A. oryzae, A. aculeatus, Talaromyces minioluteus, Hypocrea jecorina, Gliomastix murorum, Myrothecium inundatum,* and *Curvularia avinis* cultured with poor marine agar (PMA) (10).

The use of antibiotics for a certain period can cause some problem related to pathogenic bacteria resistance towards these antibiotics whether in fish or shrimp body and in addition to the residue which will pollute the environment. Besides, this will also harm humans' health by consuming contaminated sea products. Replacing antibiotics with natural compounds that have antibacterial activity against *vibrio* offers a new environmentally friendly solution. The potential of sponge-associated fungus *T. longibranchiatum* as a producer of antibacterial compounds has been examined. According to (11), the study results showed that ethyl acetate extract was able to inhibit the growth of several types of both gram-positive and negative bacteria. *Vibrio spp*. is categorized as a member of the gram-negative bacteria group. Accordingly, it is presumed that the extract is also able to inhibit its growth. This study aimed to determine the antibacterial activity of the ethyl acetate extract of sponge-associated fungus *T. longibranchiatum* as anti-vibrio.

MATERIAL AND METHODS

Fungus Isolate

The samples of isolate used in this study were sponge-associated fungi coded TE-PF-03.1. The sponges were collected from the water of Falajava Beach, Ternate Island, North Maluku, Indonesia (coordinates 00^{o}47'09.12" N; 127^{o} 23'21.76" E) at 3-30 m depth. The fungus has been investigated and identified molecularly using ITS rDNA sequence as *T. longibrachiatum* and has been morphologically confirmed (11).

Fungus Culture

Regeneration of fungus collection was carried out before the culture process (i.e. subculture for 7 days). Isolate of *T. longibranchiatum* TE-PF.03.1 was cultured using Malt Extract Agar (MEA Merck) media in several petri dishes. Preparation of MEA was carried out using sterile seawater. According to (11), the final condition of the media showed that the salinity was 60‰

and 5.6 pH. The culture was carried out for 9 days in the environmental condition with 24 hours dark, static, and 27^{o}C temperature.

Secondary Metabolites Extraction

Following 9 day-culture, the media and the micelle were cut into small pieces using a clean knife and macerated with ethyl acetate (1:1v/v). Ethyl acetate extract was obtained after filtration and evaporation processes using a rotary evaporator (40^{o}C) under low pressure. Some part of the ethyl acetate extract was separated for the initial test of its potential as anti-vibrio, while the remaining extract was partitioned using a separatory funnel with methanol (50%) and ethyl acetate (1:1v/v) solvents. Each fraction of polar (PE) and semipolar ethyl acetate (SPE) was evaporated using rotavapor to form ready to test extract as anti-vibrio.

Profiling Secondary Metabolites

The constituents compound of PE and SPE extracts were predicted using the Thin Layer Chromatography (TLC) method (12). In this process, after the extract being developed using a certain mobile phase, it will produce spots on the TLC plate and are identified using Rf (Retention factor) value. The spots on the TLC plate were visualized with 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 (12, 13). Furthermore, the TLC plate was heated at 110^{o}C for 2-3 minutes. Besides, the visualization was done by UV light (λ200-400 nm) which was equipped with an absorption profile using a UV-Vis spectrophotometer.

Antibacterial Activity Test

Vibrio bacteria used in this study were *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*. The subculture of tested bacteria was carried out in Nutrient Broth (NB) and incubated for 24 hours at 37^{o}C. The initial antibacterial bioassay was done by determining the inhibition zone diameter using a disc diffusion assay (14). *Vibrio* bacteria were cultured in Mueller-Hinton Agar (MHA) medium with 0.5 McFarland ($1.5x10^{8}$CFU mL^{-1}) initial density. The bacterial suspension was inoculated using the swab method and left it off for 15 minutes to ensure that all the suspension was absorbed by the media. Moreover, the extract preparation was started by making a stock solution with 50 mg mL^{-1} concentration in DMSO. Then, 10 µL of extract solution was dropped onto the surface of sterile disc paper (Oxoid, 6 mm diameter), and it resulted in 500 µg $disc^{-1}$ final concentration. The inhibition zone was measured after 24 hours incubation period at 37^{o}C.

Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was carried out on fractions A and B of *T. longibranchiatum* ethyl acetate extract. Broth Dilution method was used to determine MIC value referring to the method with resazurin microtiter assay (REMA) (15, 16). The first, 100 µL of the extract solution in DMSO solvent with the highest concentration (2,048 µg mL^{-1}) was filled in the first well in certain rows. Then, the next well was filled with 50 µL of sterile MHB nutrients. Moreover, the test material as much as 50 µL was transferred from the first well to the next well to achieve serial dilution (at wells number 1-11). Meanwhile, 30 µL of resazurin solution (0.02% in distilled water) was added to each well, then 10 µL of Vibrio bacterial suspension ($1x10^{8}$ CFU mL^{-1}) were added to each well. Chloramphenicol was used as a positive control (with the highest concentration of 64 µg mL^{-1}). Growth control was made

in the 12t^{h} row. Each well of this raw contained only MHB growth media without the addition of any test material. The microplate was incubated at 37^{o}C for 24 hours. After the incubation period, the well as growth control appeared pink. At last, the MIC value was determined based on the lowest concentration that could inhibit the growth of Vibrio bacteria and appeared blue.

RESULTS AND DISCUSSION

Based on the results of previous studies, *T. longibranchiatum* cultured using MEA media will reach the peak of its secondary metabolites production on day 7. At the following periods, the production decreased, but the antibacterial activity increased until the 9th day of culture. The ethyl acetate extract metabolite has antibacterial activity through the disc diffusion test (500 µg disc^{-1}) against several gram-positive and negative bacteria, while the methanol extract is inactive (11). Similar results were also found by other researchers stating that the ethyl acetate metabolite extract from endophytic fungal isolates mostly showed higher antibacterial activity than the methanol extract (17).

T. longibranchiatum sponge-associated fungus in this study was cultured using MEA for 9 days. Besides, the anti-vibrio potential of its ethyl acetate extract on disc diffusion test (500 and 250 µg disc^{-1}) can be seen in Figure 1 and it shows a clear zone appearing. This ethyl acetate extract was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus,* and *V. parahaemolyticus.*

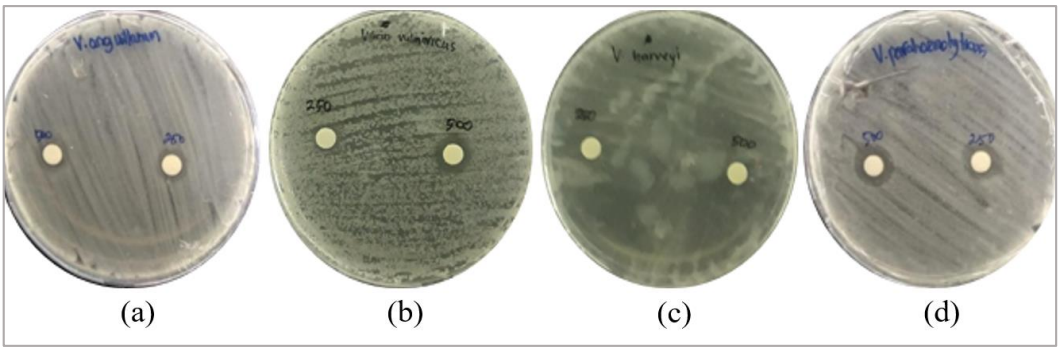

Figure 1. Anti-vibrio potential of *T. longibranchiatum* ethyl acetate extract by using disc diffusion method (500 and 250 µg disc^{-1}): (a) *V. anguillarum,* (b) *V. vulvinificus,* (c) *V. harveyi,* (d). *V. parahaemolyticus*

Further research was done to determine the polarity of the active compounds carried out by partition method using non-interfering solvents. A relatively more polar fraction was extracted using methanol (50%). The profile of the compounds in polar (PE) and semipolar (SPE) extracts corresponded to the appearance of several spots on the TLC plate as seen in Figure 2. The spots appeared fluorescent blue when being irradiated by UV indicating that the organic compound had double bonds (diene/polyene or conjugated) (18). Compounds that react positively with vanillin reagent would show certain colored spots (varying colors) characterizing the presence of carbonyl functional groups (ketones, aldehydes). These compounds probably come from phenolic, flavonoids, terpenoids, steroids, fatty acids/essential oils, or high molecular weight alcohol groups. Besides, vanillin reagent is sensitive to the

steroid class. If the compound reacts positively with the ninhydrin reagent, the compound contains nitrogen (amines, peptides, or alkaloids). Meanwhile, phenolic group compounds will react positively to ferric (III) chloride reagent (12, 19). The results of the study showed that all spots in ethyl acetate extract were not reactive to ninhydrin and ferric (III) chloride reagents; it is accordingly assumed that they are not from nitrogen or phenolic compounds. Perhaps both extract A and B are terpenoids or steroids.

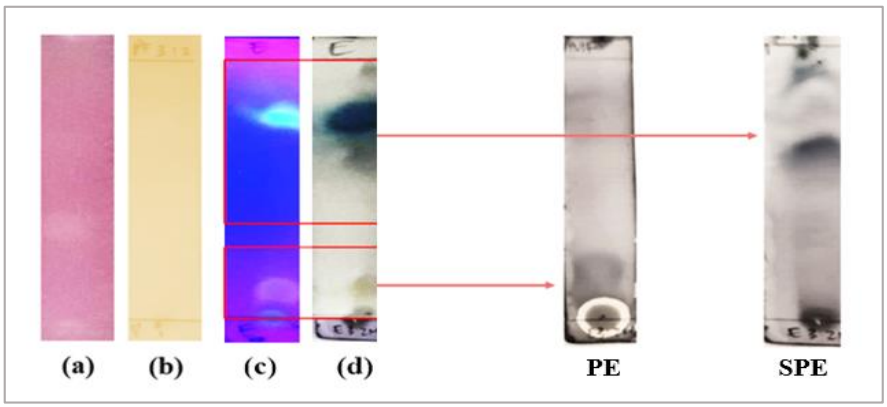

Figure 2. TLC profile of *T. longibranchiatum* ethyl acetate extract and its visualization using: (a) 0.25% ninhydrin, (b) 1% ferric (III) chloride, (c) 365 nm UV light, (d) 2% vanillin-H_2SO_4 (Note: PE=polar extract, SPE=semipolar extract)

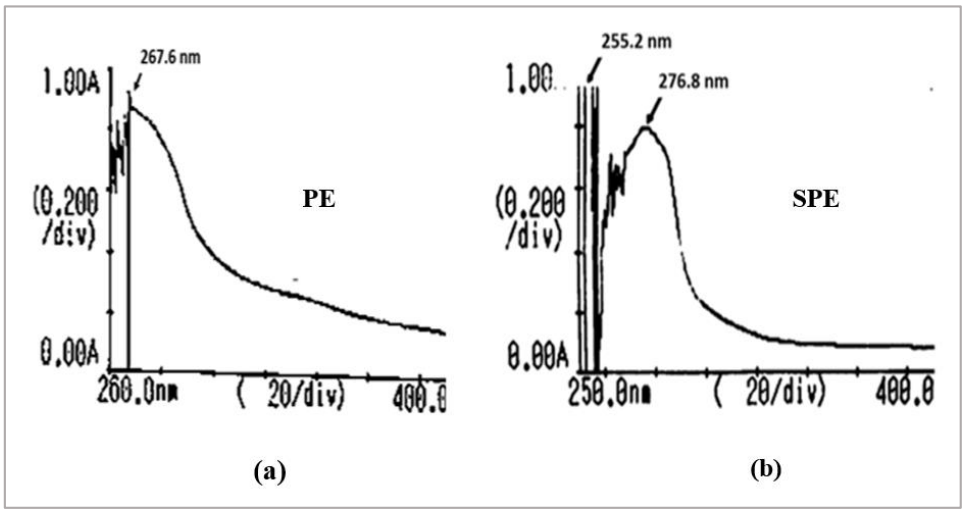

Figure 3. UV absorption spectra patterns of *T. longibranchiatum* ethyl acetate extract : (a) polar, (b) semipolar (Note: PE=polar extract, SPE=semipolar extract)

The spectra pattern of UV light absorption is shown in Figure 3. Extract PE has a pattern with 1 peak, i.e. at λ 267.6 (A=0.93), while SPE has 2 clear peaks, at λ 255.2 nm (A=4.00) and λ 276.8 (A=0.80). The probability is there were other UV absorption peaks in extract SPE. This was also reflected in the TLC profile of SPE which was observed in more than 2 spots after visualization with 2% vanillin-H_2SO_4 reagent (as in Figure 2d). Based on the literature study,

some terpenoids and steroids possess a carbonyl functional group and also conjugated double bonds. This assumption is strengthened by observing the spectra patterns of each PE and SPE. The result of previous research (20), the spectra pattern of UV light absorption from terpenoids seems to have an absorption peak at λ 259.57 nm for monoterpenoids (C10) compounds which are assumed to be thymol and at λ 260.47 nm for sesquiterpenoids (C15) which is assumed to be chiloscyphone. The structure of chiloscyphone compounds contains ketone functional groups and conjugated double bonds, whereas thymol only has conjugated bonds. This assumption was strengthened by previous statements (21), that conjugated double bond which also has a carbonyl functional group will produce chromophores that intensively absorb UV light in λ 230-270 nm range, and weakly at 300-330 nm. Meanwhile, regarding the chemical structure of steroids, 2 double bonds can be distributed between two adjoining rings (heteroannular diene). In this case, the steroid will absorb in the UV region at 220-250 nm. Moreover, it is also possible that 2 ethylene bonds ($H_2C == CH_2$) are in the same ring (homoannular dienes). Consequently, this will shift the absorption peak to 260-285 nm.

Based on the data in the following Table 1, the SPE obtained from *T. longibranchiatum* has better anti-vibrio activity than the PE. It has strongest potential against *V. vulvinificus* with a 256 μg mL^{-1} MIC value. There are only 2 species sensitive to chloramphenicol, i.e. *V. harveyi* and *V. vulvinificus*, and 2 others that are categorized in the intermediate group. The anti-vibrio potential of SPE is still weak; this is presumably because the extract is not yet pure. The extract is still in the form of a mixture of several compounds which may not be fully synergistic in supporting its antibacterial properties.

Table 1. MIC values of polar (PE) and semipolar (SPE) ethyl acetate extract against *Vibrio* spp. bacteria

Test Bacteria	MIC value against *Vibrio* spp. bacteria (μg mL^{-1})		
	PE	SPE	Chloramphenicol
V. harveyi	> 2,048	512	8
V. anguillarum	> 2,048	512	16
V. vulvinificus	1,024	256	2
V. parahaemolyticus	1,024	1,024	16

*(22) MIC>32 = resistant, 16-32 =intermediate, <8 =sensitive

Based on various results of other previous studies, there are many terpenoids isolated from fungi. *Sesquiterpenes, meroterpenes*, and *diterpenes* make up the largest proportion of *terpenes*. The genera of *Penicillium, Aspergillus*, and *Trichoderma* fungi are terpenoid's dominant producers. The majority of fungi isolated from living material from the sea (animals and plants) produce terpenoids and many exhibit various bioactivities, such as cytotoxicity, toxicity, anti-inflammatory, enzyme inhibitors, including antibacterial activities (23, 24). Similarly in butanolic extract of terrestrial *Trichoderma sp.* (isolated from forest plants), it is mostly dominated by terpenoid compounds identified as terpenes (limonene) (92.6%). Other constituents represent a small amount proportion consisting of hydrocarbons (2.01%), alcohol (2.4%), ketones (1.78%), and esters (1.03%). Culture activity using PDA media produces more

terpenoid metabolites than MEA. The growth of *S. aureus, S. epidermidis*, and *M. luteus* was inhibited at 500 μg mL^{-1} concentration, while the growth of *E. coli* was inhibited at 1 mg L^{-1} concentration (25).

The compounds in extract B were relatively less polar; as the character of terpenoids or steroids which are composed of isoprene (C5) hydrocarbon framework and were presumed to have a carbonyl or hydroxyl group. The presumption of antimicrobial properties is according to (26) stating that oxygenated terpenes show strong antibacterial activity, particularly against gram-negative bacteria. The increase in antimicrobial activity is associated with the presence of hydroxyl functional groups (phenolic compounds or alcohols), while the hydrocarbon groups produce relatively low activity. The bacteria which could be inhibited were *S. aureus, B. cereus, E. coli*, and *Salmonella enterica*. These compounds showed a high potential of antimicrobial effect. Carvacrol, l-carveol, eugenol, trans-geraniol, and thymol showed higher activity when compared to sulfanilamide. Terpineol showed excellent bactericidal activity against *S. aureus* strains. Meanwhile, carveol, citronellol, and geraniol exerted a rapid bactericidal effect against *E. coli*. The images obtained by scanning electron microscopy (SEM) show that the mechanism which causes the death of the bacterial cell is based on the loss of integrity on cellular membrane function.

The steroid group and its derivatives isolated from several strains of fungi have also been examined and the results of the study showed antimicrobial activities. Steroids are also produced by *Trichoderma sp., Penicillium sp.*, and *Acremonium sp.* with their greatest abundance respectively are ergosterol, ergostatetraenol, ergostapentaene, neoergosterol, and eburicol. Ergosterol is a sterol (alcohol steroid) commonly found in the plasma membrane of fungi. These steroids have antimicrobial activity against several gram-positive and negative bacteria, for instance. *S. aureus, Bacillus sp., B. cereus, Listeria ivanovii, E. coli, Citrobacter freundii*, and *Salmonella* spp. However, their antimicrobial activity is weak against all bacteria with highest inhibition zone (24 mm) for eburicol (27). Similar results were also seen in this study, extract B was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*, but with high MIC values, ranging from 256-1,024 μg mL^{-1}.

CONCLUSION

Semipolar ethyl acetate extract (SPE) from sponge-associated fungus *T. longibranchiatum* has stronger antibacterial activity than its polar extract (PE), so it is concluded that SPE has the potential to be developed as an anti-vibrio compound. Besides, the strongest potential of SPE was able to inhibit the growth of *V. vulvinificus* with 256 μg mL^{-1} MIC value, while the weakest was against *V. parahaemolyticus* with 1,024 μg mL^{-1} MIC value.

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(27) Dos Santos Dias A.C., Couzinet-Mossion A., Ruiz N., Lakhdar F., Etahiri S., Bertrand S., Ory L., Roussakis C., Pouchus Y.F., Nazih E.H. and Wielgosz-Collin G. Steroids from marine-derived fungi: Evaluation of antiproliferative and antimicrobial activities of eburicol. *Mar Drugs* 2019; 17(6): 372-377.

2. Manuscript sent for review #1 (21-2-2021)

Jordan Journal of Pharmaceutical Sciences

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ISSN:1995- 7157

#108326 Review

SUMMARY REVIEW EDITING

Submission

Authors	Sri Sedjati, Ambariyanto Ambariyanto, Agus Trianto, Ali Ridlo, Endang Supriyantini, Ervia Yudiati, Teguh Firmansyah
Title	Anti-Vibrio from Ethyl Acetate Extract of Sponge- Associated Fungus Trichoderma longibrachiatum
Section	Articles
Editor	Ibrahim Alabbadi

Peer Review

Round 1

Review Version	108326-131323-2-RV.DOCX 2021-02-21
Initiated	2021-02-21
Last modified	2021-03-17
Uploaded file	None
Editor Version	108326-131924-1-ED.DOCX 2021-02-21

3. Manuscript accepted editors & comment

[JJPS] Editor Decision Kotak Masuk ×

dar.journal@ju.edu.jo Kam, 1 Apr 2021 15.39
kepada saya

Inggris > Indonesia Terjemahkan pesan Nonaktifkan u

Assalamualaikum Sri Sedjati:

We have reached a decision regarding your submission to Jordan Journal of Pharmaceutical Sciences, "Anti-Vibrio from Ethyl Acetate Extract of Sponge Associated Fungus Trichoderma longibranchiatum".

Our decision is: Revisions Required

Prof. Ibrahim Alabbadi
jjps@ju.edu.jo

Reviewer A:

Does the title agree with the contents?:
yes

Is the abstract written in a proper way to reflect the results obtained?:
yes

Are the methods used suitable for this research?:
i think the procedure that used for partitioning the extract is not well explained, since the author used methanol and ethyl acetate for partitioning, these two solvents are miscible in each other, and as known the solvents used in this case must be immiscible.

Are the obtained results well presented?:
the chemical constituents of both fractions were compared only by spraying technique of the TLC, which was not clear enough, more advance technique must required as LC-MS.

Do you think that the discussions agree with the results obtained?:
Using chloramphenicol as positive control for gram negative bacteria, while it is used for gram positive bacteria must be explained

Do you think the conclusions agree with the results obtained?:
yes, but there is a need to be more specific for the cause of the differences of antibacterial activity of both extracts and the chemical constituents, after doing more analysis rather than TLC

Do the illustrations and tables agree with the nature of this research?:
figure 2 is not clear enough, to conclude the chemical constituents. Also figure 1 does not show the differences in antibacterial activity

Are the references adequate?:
yes

Is the article original?:
yes

Do you have any suggestions that might enhance the quality of the article?:
see above

Additional Comments:
No

Referee Opinion::
Accept with minor alterations

Reviewer B:

Does the title agree with the contents?:
yes

Is the abstract written in a proper way to reflect the results obtained?:
yes

Are the methods used suitable for this research?:
yes

Are the obtained results well presented?:
yes

Do you think that the discussions agree with the results obtained?:
yes

Do you think the conclusions agree with the results obtained?:
yes

Do the illustrations and tables agree with the nature of this research?:
yes

Are the references adequate?:
yes

Is the article original?:
yes

Do you have any suggestions that might enhance the quality of the article?:
more techniques could be used like HPLC, GC-MS to identify the constituents of extract

Additional Comments:
see attachment

Referee Opinion::
Accept with minor alterations

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4. Revision #1submission
   - Submit Revision (5-5-2021)

Sri Sedjati <srisedjati01@gmail.com> 5 Mei 2021 08.43
kepada Jordan

Dear Prof. Ibrahim Alabbadi

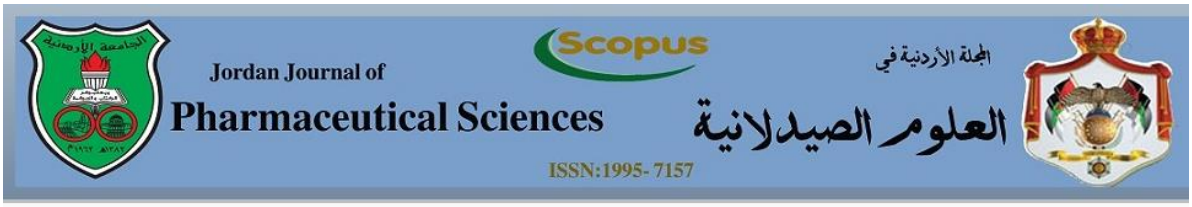
Assalamualaikum.

I have uploaded the revised version on the system.

Thank you for the attention to my manuscript.

Wassalamualaikum.

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



#108326 Review

SUMMARY REVIEW EDITING

Submission

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Editor Version	108326-131924-1-ED.DOCX 2021-02-21 108326-131924-2-ED.DOCX 2021-05-21
Author Version	108326-133324-1-ED.DOCX 2021-05-05

- Responses for reviewer A (5-5-2021)

Response to reviewers

Reviewer A:

Does the title agree with the contents?:
yes

Is the abstract written in a proper way to reflect the results obtained?:
yes

Are the methods used suitable for this research?:
i think the procedure that used for partitioning the extract is not well explained, since the author used methanol and ethyl acetate for partitioning, these two solvents are miscible in each other, and as known the solvents used in this case must be immiscible.

- Methanol is added water to increase its polarity, so that it can be separated with ethyl acetate. Extract was partitioned using a separatory funnel with water-methanol 70:30 and ethyl acetate (1:1v/v) solvents

Are the obtained results well presented?:
the chemical constituents of both fractions were compared only by spraying technique of the TLC, which was not clear enough, more advance technique must required as LC-MS.

- I haven't done an analyst using LC-MS

Do you think that the discussions agree with the results obtained?:
Using chloramphenicol as positive control for gram negative bacteria, while it is used for gram positive bacteria must be explained

- Chloramphenicol is an antibacterial agent with a broad spectrum of activity against gram-positive bacteria, gram-negative bacteria.

Do you think the conclusions agree with the results obtained?:
yes, but there is a need to be more specific for the cause of the differences of antibacterial activity of both extracts and the chemical constituents, after doing more analysis rather than TLC

Do the illustrations and tables agree with the nature of this research?:
figure 2 is not clear enough, to conclude the chemical constituents. Also figure 1 does not show the differences in antibacterial activity

- I don't have a clearer picture, so I replaced it in the form of a table

Are the references adequate?:
yes

Is the article original?:
yes

Do you have any suggestions that might enhance the quality of the article?:
see above

- Responses for reviewer B (5-5-2021)

Response to reviewers

Reviewer B:

Does the title agree with the contents?:
yes

Is the abstract written in a proper way to reflect the results obtained?:
yes

Are the methods used suitable for this research?:
yes

Are the obtained results well presented?:
yes

Do you think that the discussions agree with the results obtained?:
yes

Do you think the conclusions agree with the results obtained?:
yes

Do the illustrations and tables agree with the nature of this research?:
yes

Are the references adequate?:
yes

Is the article original?:
yes

Do you have any suggestions that might enhance the quality of the article?:
more techniques could be used like HPLC, GC-MS to identify the constituents of extract

- I haven't done an analyst using HPLC or GC-MS, hopefully it can be done in the next stage of research.

- Revised manuscript 1 (5-5-2021)

Anti-Vibrio from Ethyl Acetate Extract of Sponge-Associated Fungus *Trichoderma longibra~~n~~chiatum*

ABSTRACT

Some of *Vibrio* spp. bacteria are pathogenic to humans and other organisms, including cultured fish or shrimp. This study aimed to determine the activity of ethyl acetate extract of *Trichoderma longibra~~n~~chiatum* as an anti-vibrio fungus. The test bacteria used were: *Vibrio harveyi, Vibrio anguillarum, Vibrio vulvinificus,* and *Vibrio parahaemolyticus*. The fungus was cultured using Malt Extract Agar (MEA) medium for 9 days at 27ºC (static conditions, 24 hours dark, pH 5.6, and salinity 60 ppt). Extracts obtained by maceration using ethyl acetate, then the extract is partitioned using water-methanol 70:30 and ethyl acetate. Each fraction was concentrated to obtain polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extracts. The components of the constituent extract were traced with a Thin Layer Chromatography (TLC) and followed by ultraviolet spectrophotometry. The anti-vibrio activity was determined based on the value of Minimum Inhibitory Concentration (MIC). The results of the study showed that SPE was more potential to be used as anti-vibrio. The strongest activity was able to inhibit the growth of *Vibrio vulvinificus* with 256 µg mL^{-1} MIC value, while the weakest was against *Vibrio parahaemolyticus* with 1.024 µg mL^{-1} MIC value. In conclusion, SPE has the potential to be developed as an anti-vibrio compound, particularly against *Vibrio vulvinificus*.

Keywords: Semipolar-ethyl acetate extract, Minimum Inhibitory Concentration, *Vibrio vulvinificus*

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INTRODUCTION

Vibrio is a genus of bacteria found in a variety of freshwater and marine habitats. There are more than 100 species of *Vibrio* spp., and 12 of which cause infection in humans. *Vibrio cholerae* (*V. cholerae*) can cause cholera, a severe diarrheal disease that can be transmitted through contaminated water. Non-cholera *Vibrio* spp., such as *V.*

parahaemolyticus, V. alginolyticus, and *V. vulnificus,* may cause vibriosis, an infection with various clinical expressions, and the mildest of which is gastroenteritis. Vibriosis infection is usually caused by exposure to seawater or by consuming contaminated raw or undercooked seafood. There is an exception, *V. vulnificus* which is an opportunistic pathogen causing wound infection which can rapidly lead to septicemia (1, 2). Infections caused by *V. vulnificus* are generally fatal. Therefore, accurate diagnosis and direct treatment are very important since the infection may cause death to the sufferer (3). antibiotics are widely used to treat vibriosis. The commonly used antibiotics include doxycycline, quinolone, cephalosporin, ciprofloxacin, tigecycline, and ceftazidime (2, 3). *Vibrio* spp. are gram-negative bacteria that causes vibriosis in humans and animals. Moreover, fishery products and post-harvest products are common intermediaries for the transfer of bacteria from their original habitat to their new hosts (5).

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•I've added it.

The fisheries sector is one of the most fundamental fields for a country's food security. On the other hand, fisheries systems also play a major role in spreading vibriosis to humans. Vibriosis is a bacterial disease reported in Indonesian marine fish culture since the 1990s. The disease is reported mostly found in grouper (*Epinephelus* sp.) and shrimp (*Penaeus monodon*, *Litopenaeus vannamei*) culture, although

infection also occurs in snapper (*Lates calcarifer*) and abalone (*Haliotis squamata*) culture. The agent causing vibriosis in marine fish in Indonesia involves several vibrio species, including *V. harveyi, V. anguillarum, V. alginoluticus, V. parahaemolyticus* (4). Some efforts are done to control vibriosis in fish farming activities still rely on the use of drugs or antibiotics given through oral and immersion.However, feed residues will cause pollution in the waters due to antibiotic contamination. Nowadays, several Vibrio species are even resistant to certain types of antibiotics. Based on the research (5) conducted on the North Coast of Java found that *V. parahaemolyticus* contaminating vanname shrimp is resistant to erythromycin (90%), amoxicillin-clavulanic acid (83.33%), and nitrofurantoin (58.33%).

Vibrio spp. live in marine habitats along with other bacterial species and microorganisms, like fungi. Competition among the inhabitants of an ecosystem will occur to compete for space and nutrition. Consequently, there is a possibility that there are any antagonistic species, both within among themselves and towards other species. Some research resulted in the fact that several marine-derived fungi species synthesize secondary metabolites of anti-vibrio as their chemical weapons to compete and to avoid predation. Several anti-vibrio metabolites found are such as ethyl acetate extract from sponge-associated fungus *Tricoderma asperellum* (6);

Commented [A5]: Trichoderma

prenylxanthone and aspergixanthones from the marine-derived fungus *Aspergillus sp*. cultured with shaken Czapek-Dox media (7); indole-diterpenoids and steroids isolated from *Penicillium janthinellum* (8), secondary metabolites of the mangrove-associated fungus *Aspergillus sp*. (9), and secondary metabolites of marine invertebrates associated fungi *A. flavus, A. oryzae, A. aculeatus, Talaromyces minioluteus, Hypocrea jecorina, Gliomastix murorum, Myrothecium inundatum,* and *Curvularia avinis* cultured with Poor Marine Agar (PMA) (10).

The use of antibiotics for a certain period can cause some problem related to pathogenic bacteria resistance towards these antibiotics whether in fish or shrimp body and in addition to the residue which will pollute the environment. Besides, this will also harm humans' health by consuming contaminated sea products. Replacing antibiotics with natural compounds that have antibacterial activity against *Vibrio* spp. offers a new environmentally friendly solution. The potential of sponge-associated fungus *T. longibra~~n~~chiatum* as a producer of antibacterial compounds has been examined. According to (11), the study results showed that ethyl acetate extract was able to inhibit the growth of several types of both gram-positive and negative bacteria. *Vibrio ~~spp.~~* is categorized as a member of the gram-negative bacteria group. Accordingly, it is presumed that the extract is

also able to inhibit its growth. This study aimed to determine the antibacterial activity of the ethyl acetate extract of sponge-associated fungus *T. longibrachiatum* as anti-vibrio.

MATERIAL AND METHODS

Fungus Isolate

The samples of isolate used in this study were sponge-associated fungi coded TE-PF-03.1. The sponges were collected from the waters of Falajava Beach, Ternate Island, North Maluku, Indonesia (coordinates 00º47'09.12" N; 127º 23'21.76" E) at 3-30 m depth. The fungus has been investigated and identified molecularly using Internal Transcribed Spacer (ITS) rDNA sequence as *T. longibrachiatum* and has been morphologically confirmed (11).

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Fungus Culture

Regeneration of fungus collection was carried out before the culture process (i.e. subculture for 7 days). Isolate of *T. longibrachiatum* TE-PF.03.1 was cultured using Malt Extract Agar (MEA Merck) media in 20 Petri dishes. Preparation of MEA was carried out using sterile seawater. According to (11), the final condition of the media showed that the salinity was 60 ppt and pH 5.6. The culture was carried out for 9 days in the environmental condition with 24 hours dark, static, and 27ºC temperature.

Secondary Metabolites Extraction

Following 9 day-culture, the media and the micelle were cut into small pieces and macerated with ethyl acetate (1:1v/v). Ethyl acetate extract was obtained after filtration and evaporation processes using a rotary evaporator (40ºC) under low pressure. Some part of the ethyl acetate extract was separated for the initial test of its potential as anti-vibrio, while the remaining extract was partitioned using a separatory funnel with water-methanol 70:30 and ethyl acetate (1:1v/v) solvents. Each fraction of polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extract was concentrated and ready to antibacterial test as anti-vibrio (11).

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•I've added it.

Profiling Secondary Metabolites

The constituents compound of PE and SPE extracts were predicted using the Thin Layer Chromatography (TLC) method (12). In this process, after the extract being developed using a certain mobile phase, it will produce spots on the TLC plate and are identified using Rf (Retention factor) value. The spots on the TLC plate were visualized with ultraviolet (UV) light 365 nm, 2% vanillin-H_2SO_4, 0.25% ninhydrin in acetone, and 1% ferric (III) chloride in methanol (12, 13). Furthermore, the TLC plate was heated at 110ºC for 2-3 minutes. Besides, the visualization was done by UV light (λ200-400 nm) which

was equipped with an absorption profile using a UV-Vis spectrophotometer.

Antibacterial Activity Test

Vibrio bacteria used in this study were *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*. The subculture of tested bacteria was carried out in Mueller-Hinton Broth (MHB; Oxoid) and incubated for 24 hours at 37^{o}C. The initial antibacterial bioassay was done by determining the inhibition zone diameter using a disc diffusion assay (14). *Vibrio* bacteria were inoculated in Mueller-Hinton Agar (MHA; Oxoid) media with 0.5 McFarland (1.5x10^{8}CFU mL^{-1}) initial density using the swab method. Moreover, the extract preparation was started by making a solution with 50 and 25 mg mL^{-1} concentration in DMSO. Then, 10 µL of extract solution was dropped onto the surface of sterile disc paper (Oxoid, 6 mm diameter), and it resulted in 500 and 250 µg disc^{-1} final concentration. The inhibition zone was measured after 24 hours incubation period at 37^{o}C.

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Minimum Inhibitory Concentration Test

Minimum Inhibitory Concentration (MIC) test was carried out on fractions PE and SPE of *T. longibra~~n~~chiatum* ethyl acetate extract. Broth Dilution method was used to determine MIC value referring to the method with resazurin microtiter assay (REMA) (15, 16). The first,

100 µL of the extract solution in DMSO solvent with the highest concentration was filled in the first well in certain rows. Then, the next well was filled with 50 µL of sterile MHB . Moreover, the test material as much as 50 µL was transferred from the first well to the next well to achieve 10 series of dilution (2,048-4 µg mL^{-1}) . Meanwhile, 30 µL of resazurin solution (Sigma-Aldrich, 0.02% in distilled water) was added to each well, then 10 µL of Vibrio bacterial suspension (1.5 $x10^8$ CFU mL^{-1}) were added to each well. Chloramphenicol was used as a positive control (with series concentrations from 64-0.125 µg mL^{-1}). Well the 11th filled DMSO as a negative control and the 12th filled MHB as media control. The microplate was incubated at 37ºC for 24 hours. After the incubation period, the well as growth control appeared pink. At last, the MIC value was determined based on the lowest concentration that could inhibit the growth of *Vibrio* spp. and appeared blue.

Commented [A9]: Did you mean Muller Hinton Broth? Moreover, what is the source of this medium?

–I've mentioned it in the previous paragraph.

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RESULTS AND DISCUSSION

Based on the results of previous studies, *T. longibranchiatum* cultured using MEA media will reach the peak of its secondary metabolites production on day 7. At the following periods, the production decreased, but the antibacterial activity increased until the

9th day of culture. The ethyl acetate extract metabolite has antibacterial activity through the disc diffusion test (500 µg $disc^{-1}$) against several gram-positive and negative bacteria, while the methanol extract is inactive (11). Similar results were also found by other researchers stating that the ethyl acetate metabolite extract from endophytic fungal isolates mostly showed higher antibacterial activity than the methanol extract (17).

T. longibrachiatum sponge-associated fungus in this study was cultured using MEA for 9 days. Besides, the anti-vibrio potential of its ethyl acetate extract on disc diffusion test (500 and 250 µg $disc^{-1}$) can be seen in Table 1 and it shows a inhibition zone formed. This ethyl acetate extract was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus,* and *V. parahaemolyticus.*

Commented [A11]: I can not see the activity in plate C.. its not clear.
Furthermore, as I see in this figure, the activty is too low.
Do you think that your tested bacteria are sensitive to this extract.
•I don't have a clearer picture, so I replaced it in the form of a table.

Table 1. Anti-vibrio potential of *T. longibrachiatum* ethyl acetate extract by using disc diffusion method

Tested Bacteria	Concentration of extracts (µg $disc^{-1}$)	
	500	250
V. harveyi	+	-
V. anguillarum	+	+
V. vulvinificus	+	+
V. parahaemolyticus	+	+

Note: += inhibition zone formed, - = no inhibition zone

Further research was done to determine the polarity of the active compounds carried out by partition method using immiscible solvents. Methanol is added water to increase its polarity, so that it can be separated with ethyl acetate. The profile of the compounds in PE and SPE extracts corresponded to the appearance of several spots on the TLC plate as seen in Figure 1. The spots appeared fluorescent blue when being irradiated by UV indicating that the organic compound had double bonds (diene/polyene or conjugated) (18). Compounds that react positively with vanillin reagent would show certain colored spots (varying colors) characterizing the presence of carbonyl functional groups (ketones, aldehydes). These compounds probably come from phenolic, flavonoids, terpenoids, steroids, fatty acids/essential oils, or high molecular weight alcohol groups. Besides, vanillin reagent is sensitive to the steroid class. If the compound reacts positively with the ninhydrin reagent, the compound contains nitrogen (amines, peptides, or alkaloids). Meanwhile, phenolic group compounds will react positively to ferric (III) chloride reagent (12, 19). The results of the study showed that all spots in ethyl acetate extract were not reactive to ninhydrin and ferric (III) chloride reagents; it is accordingly assumed that they are not from nitrogen or phenolic compounds. Perhaps both extract PE and SPE are terpenoids or steroids.

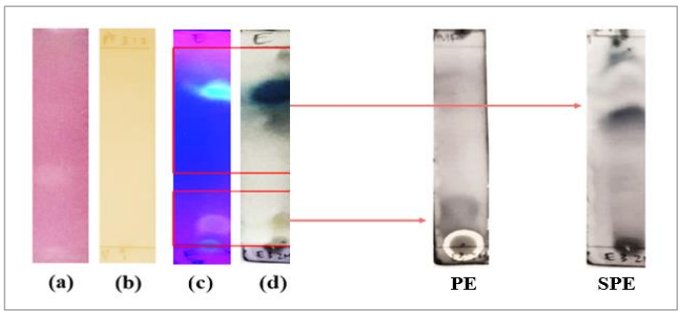

Figure 1. TLC profile of *T. longibranchiatum* ethyl acetate extract and its visualization using: (a) 0.25% ninhydrin, (b) 1% ferric (III) chloride, (c) UV light 365 nm, (d) 2% vanillin-H_2SO_4 (Note: PE=polar-ethyl acetate, SPE=semipolar-ethyl acetate)

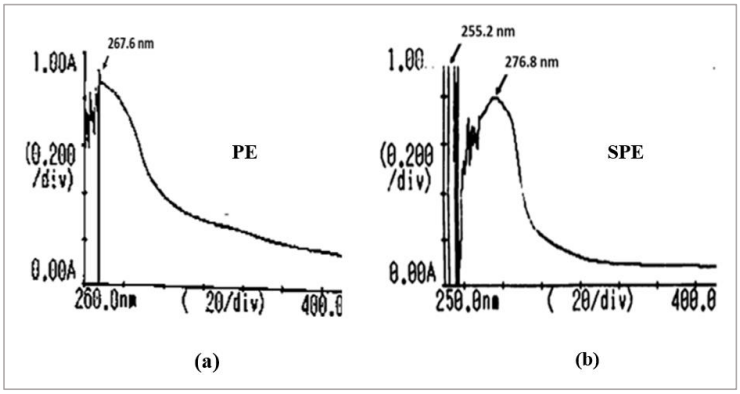

Figure 2. UV spectra (λ200-400 nm) of *T. longibranchiatum* ethyl acetate extract: (a) PE=polar-ethyl acetate , (b) SPE=semipolar-ethyl acetate)

The spectra of UV light absorption is shown in Figure 2. Extract PE has a pattern with 1 peak, i.e. at λ 267.6 (A=0.93), while SPE has 2 clear peaks, at λ 255.2 nm (A=4.00) and λ 276.8 (A=0.80). There

may be other peaks in SPE. This was also reflected in the TLC profile of SPE which was observed in more than 2 spots after visualization with 2% vanillin-H_2SO_4 (as in Figure 1d). Based on the literature study, some terpenoids and steroids possess a carbonyl functional group and also conjugated double bonds. This assumption is strengthened by observing the UV spectra patterns of each PE and SPE. The result of previous research (20), the UV spectra of terpenoids seems to have an absorption peak at λ 259.57 nm for monoterpenoids (C10) compounds which are assumed to be thymol and at λ 260.47 nm for sesquiterpenoids (C15) which is assumed to be chiloscyphone. The structure of chiloscyphone compounds contains ketone functional groups and conjugated double bonds, whereas thymol only has conjugated bonds. This assumption was strengthened by previous statements (21), that conjugated double bond which also has a carbonyl functional group will produce chromophores that intensively absorb UV light in λ 230-270 nm range, and weakly at 300-330 nm. Meanwhile, regarding the chemical structure of steroids, 2 double bonds can be distributed between two adjoining rings (heteroannular diene). In this case, the steroid will absorb in the UV region at 220-250 nm. Moreover, it is also possible that 2 ethylene bonds ($H_2C = CH_2$) are in the same ring (homoannular dienes). Consequently, this will shift the absorption peak to 260-285 nm.

Based on the data in the following Table 2, the SPE obtained from *T. longibrachiatum* has better anti-vibrio activity than the PE. It has strongest potential against *V. vulvinificus* with a 256 µg mL^{-1} MIC value. There are only 2 species sensitive to chloramphenicol, i.e. *V. harveyi* and *V. vulvinificus*, and 2 others that are categorized in the intermediate group. The anti-vibrio potential of SPE is still weak; this is presumably because the extract is not yet pure. The extract is still in the form of a mixture of several compounds which may not be fully synergistic in supporting its antibacterial properties.

Table 2. MIC values of polar-ethyl acetate (PE) and semipolar-ethyl acetate (SPE) extracts against *Vibrio* spp.

Tested Bacteria	MIC value against *Vibrio* spp. (µg mL^{-1})		
	PE	SPE	Chloramphenicol*
V. harveyi	> 2,048	512	8
V. anguillarum	> 2,048	512	16
V. vulvinificus	1,024	256	2
V. parahaemolyticus	1,024	1,024	16

Note: * Categories on organism as susceptible (22):
MIC>32 = resistant, 16-32 =intermediate, <8 =sensitive

Commented [A12]: Are these MIC values for Chloramphenicol against Vibrio Spp.?
Please write them I clear way
•I've corrected it.

Based on various results of other previous studies, there are many terpenoids isolated from fungi. *Sesquiterpenes, meroterpenes*, and *diterpenes* make up the largest proportion of *terpenes*. The genera of *Penicillium, Aspergillus*, and *Trichoderma* fungi are

terpenoid's dominant producers. The majority of fungi isolated from living material from the sea (animals and plants) produce terpenoids and many exhibit various bioactivities, such as cytotoxicity, anti-inflammatory, enzyme inhibitors, including antibacterial activities (23, 24). Similarly in butanolic extract of terrestrial *Trichoderma sp.* (isolated from forest plants), it is mostly dominated by terpenoid compounds identified as terpenes (limonene) (92.6%). Other constituents represent a small amount proportion consisting of hydrocarbons (2.01%), alcohol (2.4%), ketones (1.78%), and esters (1.03%). Culture activity using Potato Dextrose Agar (PDA) media produces more terpenoid metabolites than MEA. The growth of *Staphylococcus aureus, Staphylococcus epidermidis,* and *Micrococcus luteus* was inhibited at 500 µg mL^{-1} concentration, while the growth of *Escherichia coli* was inhibited at 1 mg L^{-1} concentration (25).

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The compounds in extract SPE were relatively less polar; as the character of terpenoids or steroids which are composed of isoprene (C5) hydrocarbon and were presumed to have a carbonyl or hydroxyl group. The presumption of antimicrobial properties is according to (26) stating that oxygenated terpenes show strong antibacterial activity, particularly against gram-negative bacteria. The increase in antimicrobial activity is associated with the presence of hydroxyl

functional groups (phenolic compounds or alcohols), while the hydrocarbon groups produce relatively low activity. The bacteria which could be inhibited were *S. aureus, Bacillus cereus, E. coli,* and *Salmonella enterica*. Some oxygenated terpene compounds such as carvacrol, l-carveol, eugenol, trans-geraniol, and thymol showed higher activity when compared to sulfanilamide. Terpineol showed excellent bactericidal activity against *S. aureus* strains. Meanwhile, carveol, citronellol, and geraniol exerted a rapid bactericidal effect against *E. coli*. The images obtained by scanning electron microscopy (SEM) show that the mechanism which causes the death of the bacterial cell is based on the loss of integrity on cellular membrane function.

Commented [A16]: Full name please

The steroid group and its derivatives isolated from several strains of fungi have also been examined and the results of the study showed antimicrobial activities. Steroids are also produced by *Trichoderma sp., Penicillium sp.,* and *Acremonium sp.* with their greatest abundance respectively are ergosterol, ergostatetraenol, ergostapentaene, neoergosterol, and eburicol. Ergosterol is a sterol (alcohol steroid) commonly found in the plasma membrane of fungi. These steroids have antimicrobial activity against several gram-positive and negative bacteria, for instance. *S. aureus, Bacillus sp., B. cereus, Listeria ivanovii, E. coli, Citrobacter freundii*, and

Salmonella spp. However, their antimicrobial activity is weak against all bacteria with highest inhibition zone for eburicol (24 mm) (27). Similar results were also seen in this study, extract SPE was able to inhibit the growth of *V. harveyi, V. anguillarum, V. vulvinificus*, and *V. parahaemolyticus*, but with high MIC values, ranging from 256-1,024 µg mL^{-1}.

CONCLUSION

Semipolar-ethyl acetate (SPE) extract from sponge-associated fungus *T. longibrachiatum* has stronger antibacterial activity than its polar- ethyl acetate (PE), so it is concluded that SPE has the potential to be developed as an anti-vibrio compound. Besides, the strongest potential of SPE was able to inhibit the growth of *V. vulvinificus* with 256 µg mL^{-1} MIC value, while the weakest was against *V. parahaemolyticus* with 1,024 µg mL^{-1} MIC value.

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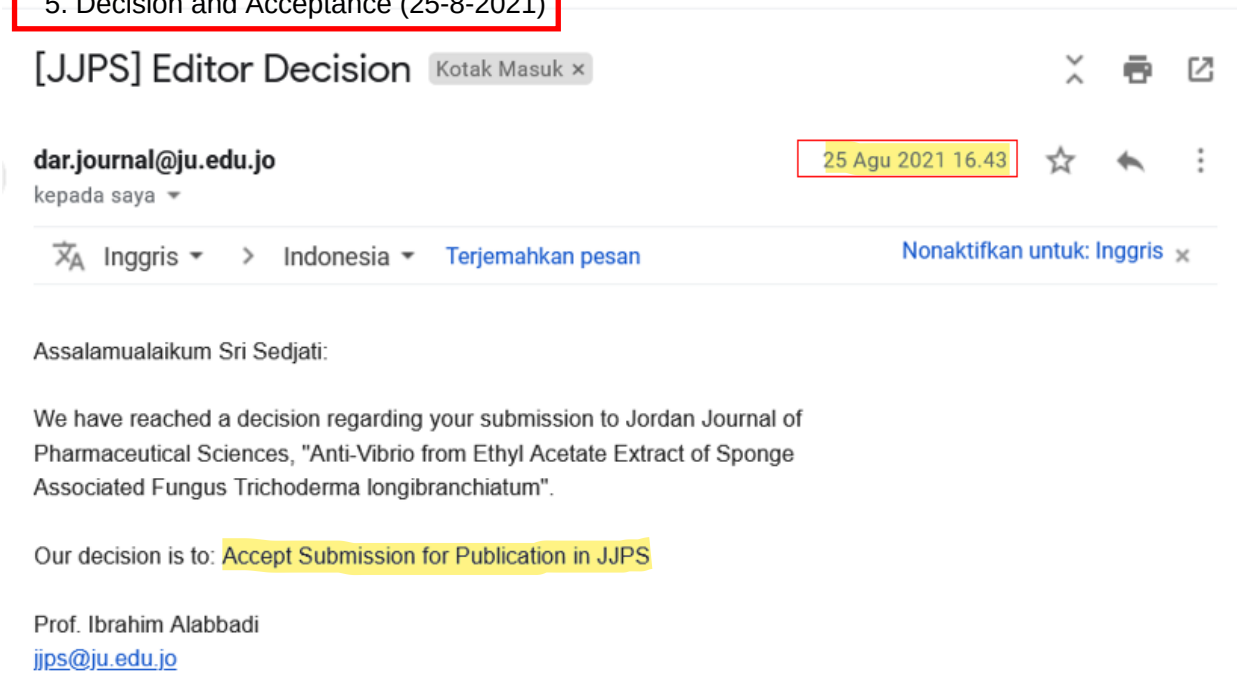

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- Revised proofread: Abstract

المضادة للفيبريو من استخراج خلات إيثيل من جمعية الفطر-الإسفنج *Trichoderma longibrachiatum*

سري سيدجاتيو[1*3]، أمبرياتو أمبرياتو[1,2]، أعوس تريانتو[1,2]، علي ريدلو[1]، إندانغ سوبريانتيني[1]، إرفيا يودياني[1]، و تيغوه فيرماندياه[2]

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تجريدي

بعض أنواع البكتيريا *Vibrio* spp. هي مسببات الأمراض للبشر والكائنات الحية الأخرى، بما في ذلك الأسماك المستزرعة أو الروبيان. هدفت هذه الدراسة إلى تحديد فعالية مستخلص أسيتات الإيثيل من *Trichoderma longibrachiatum* مثالالمضادة لVibrio spp. كانت بكتيريا الاختبار المستخدمة هي: *Vibrio harveyi* , *Vibrio anguillarum* , *Vibrio vulvinificus* , *Vibrio parahaemolyticus*. تمت زراعة الفطر باستخدام وسط مستخلص الشعير لمدة 9 أيام عند 27 درجة مئوية (ظروف ثابتة، 24 ساعة مظلمة، درجة الحموضة 5.6، و الملوحة 60 لكل ألف). يتم الاستخراج الأولي عن طريق التكسير باستخدام خلات الإيثيل ، ثم تتركز. يتم تقسيم المقتطف مع المذيبات الميثانول أكواديس (70:30) وخلات الإيثيل بنسبة 1:1. القطبية الإيثيل. ويتركز كل جزء بحيث يتم الحصول على مقتطفات خلات الإيثيل القطبي ومستخلصات خلات شبه القطبية الإيثيل. يتم تتبع مكونات المركبات المكونة لها باستخدام طريقة الكروماتوجرافيا طبقة رقيقة. نشاطالمضادة ل Vibrio يتم تحديده على أساس الحد الأدنى لقيمة التركيز المثبطة باستخدام اختبار ريسازورين. وأظهرت النتائج أن مقتطفات خلات شبه القطبية الإيثيلية هي أكثر قدره على مكافحة Vibrio. أقوى نشاط لها قادر على منع نمو V. vulvinificus مع الحد الأدنى من قيمة تركيز المثبطة من 256 جزء في المليون، في حين أن أضعف ضد V. parahaemolyticus بقيمة 1024 جزء في المليون. المركبات التي يعتقد أنها بمثابة المضادة للفيبريو هي تيربينويدات أو المنشطات. في الختام، يمكن تطوير خلات استخراج الإيثيل شبه القطبي كمركب مضاد للفيبريو، وخاصة ضد V. vulvinificus.

الكلمات الرئيسية: استخراج خلات شبه القطبية الإيثيلية، الحد الأدنى من التركيز المثبط، *Vibrio vulvinificus*

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