

Antimicrobial Activity of Fungal Extract of The *Aspergillus flavus* from Hiri Island, North Maluku to Pathogenic Bacteria

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Abstract

Antibacterial compounds from sponge association fungi are one of the alternatives to search for new antibiotics against resistant bacteria. This study aims to explore the secondary metabolites potential from sponge association fungi as MDR pathogens antibacterial and to cultivate these fungi using a variety of cultivation media. TE-BO-09.1. Isolate can inhibit 3 bacteria, *K. pneumoniae*, *B subtilis*, and *S. aureus*, but is not able to inhibit *E. coli* and *P. aeruginosa*. Cultivation using standard media of Malt Extract Agar (MEA) and media modified from fish broth (M1, M2) and cassava infusion (M3, M4) produce secondary metabolites with varying quantities and antimicrobial activity. The inhibition zone of the produced extract with 500 µg/disc concentration ranged from 7.14 to 10.32 mm. The strongest potential was shown by ethyl acetate extract from isolates cultured with M2 (9 days cultivation), of which is able to produce 10.32 mm inhibition zones against *S. aureus* and methanol extracts from isolates cultured with M4 (6 days cultivation), and able to produce 10.05 mm inhibition zones against *K. pneumoniae*. In conclusion, the fungus *Aspergillus flavus* can be culture using fish broth added glucose and cassava infusion water added peptone media to potentially produce antibacterial compounds against MDR pathogens.

Keywords : secondary metabolites; antibacterial; association fungi; MDR pathogens

INTRODUCTION

More than 70% of hospital infections are caused by bacteria that are resistant to at least 1 type of antibiotic, and some of which are even resistant to 3 or more antibiotics (multi-drug resistant/MDR) as stated by Krzowska-Firych *et al.*, (2014). These bacteria can be found in gram-positive groups (*vancomycin - resistant enterococci / VRE*, *methicillin-resistant Staphylococcus aureus / MRSA*) and gram-negative (*Klebsiella spp.*, *Acinetobacter spp.*, *Pseudomonas spp.*). The infectious bacteria which are often identified are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*,

Pseudomonas aeruginosa, and *Enterobacter spp.* known as "ESKAPE pathogens". Other bacteria are also often infectious, i.e. *Streptococcus spp.*, *Acinetobacter spp.*, *Enterococci*, *Negative Stagylococcus Coagulase*, *Bacillus cereus*, *Legionella spp.*, *Proteus mirabilis*, *Escherichia coli*, *Serratia marcescens*. (Bereket *et al.*, 2012; Bologna *et al.*, 2013; Ahmad, 2015; Almasaudi, 2015 ; Bouguenoun *et al.*, 2016; Miao *et al.*, 2017). Abebe *et al.* (2016) summarizes some molecular mechanisms which trigger bacterial resistance, they are: (a) bacterial enzymes which hydrolyze antibiotics to be inactive, (b) changing antibiotic targets (mutations of bacterial genes), (c) preventing antibiotic permeation on target

microorganisms, (d) antibiotic extracellular secretions (e.g. by using *active efflux pump*), and (e) forming biofilm.

Antibiotic resistance is a public health problem faced by many countries, including Indonesia (Kemenkes RI, English term: Ministry of Health of The Republic of Indonesia, 2011). Antibiotic resistance occurs when microorganisms undergo changes that cause the drugs used to cure the infections to become ineffective. Antibiotics are well known as drugs that are used to treat bacterial infections. Antibiotics can be bactericidal (killing bacteria) or bacteriostatic (preventing bacterial proliferation) (Kemenkes RI, 2015). Resistant microorganisms prevention can be solved by finding new antibiotics that can kill the bacteria. Moreover, one alternative source of new antibiotics is derived from natural ingredients, including from marine biological materials.

Some fungi from Indonesian waters are scientifically proven to contain antibacterial compounds. Sponge association fungus from Riung waters (East Nusa Tenggara) has the potential to produce antibacterial compounds of MDR *S. aureus* and *E. coli* (Wittriansyah, *et al.*, 2016). Endophytic fungi from *Haliclona fascigera* living in Mandeh Island waters (West Sumatra) produces ethyl acetate extracts that can inhibit bacteria *S. aureus* and *E. coli* (Handayani *et al.*, 2015). *Plakotis melobesioide* sponge association fungus from Malalayang waters (North Sulawesi) also has the potential to produce new antibacterial as well. Its methanol extract contains semi-polar bioactive compounds that can inhibit the growth of *S. aureus* bacteria better than *chloramphenicol* (Sihombing *et al.*, 2017). Several other researchers have also succeeded in isolating and identifying active compounds, such as *Trichoderma asperellum* and *T. reesei* fungus from sponge *Cinachyrella sp.* which produce antibacterial *E. coli* strain MDR (Sibero *et al.*, 2017); *Aspergillus sydowii* fungus from sponge *Axinella sp.* produces antibacterial *S. aureus* and *E. coli* MDR strains (Trianto *et al.*, 2017) and *T. saturnisporum* fungus from Pandang Island waters (North Sumatra) produces antibacterial *Staphylococcus enterica* MDR strain (Sibero *et al.*, 2018).

A study conducted by Zhou *et al.* (2016) showed that secondary metabolites of marine fungi from different habitats would produce compounds with different activities. Another factor is the difference in cultivation media as Ozkaya *et al.* (2018) proved that sponge association fungi which were cultivated in different media turned out to produce different secondary metabolites, both in their types and activities. The purpose of this study is to find out the potential of secondary metabolites of sponge association fungus as an antibacterial and to cultivate the fungus using various cultivation media. It is expected that relatively inexpensive media can produce metabolites with high bioactivity against pathogenic bacteria.

MATERIALS AND METHODS

The sample was isolated from a sponge obtained from western Hiri Island waters (Big O with coordinates 00°53'47.77"N 127°18'16.61"E), North Maluku, Indonesia (as seen in Figure 1). The fungus isolate with code TE-BO-09.1 has been identified in previous antibacterial screening research. Molecular identification of ITS rDNA sequences showed that the fungus was identified as *Aspergillus flavus* (Trianto *et al.*, 2020).

Morphological Characterization of Fungus

Active fungal isolates were cultivated using MEA media (Merck) for 7 days (at 27°C incubation temperature) for macroscopic observation of the colony shape. Furthermore, the microscopic characteristics were determined based on the *slide culture* method (Maurya *et al.*, 2011; Sibero *et al.*, 2017) by administering *Lactophenol Blue* and then were observed using a microscope with 100 times magnification.

Antimicrobial Activity Screening

Initial antibacterial screening or also called as antagonistic testing was carried out using agar plug method (Trianto *et al.*, 2017; Sibero *et al.*, 2018). Besides, the fungal association was cultured in MEA media for 7 days at 27°C temperature. The growing mycelia were then cut in circular shape with

a 1cm diameter. Furthermore, pathogenic bacteria were cultured in MHA media for antagonistic testing. Test bacteria used in this study were *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, *E. coli* and *B. cereus* which are MDR strains. The test bacteria were obtained from the laboratory of Diponegoro National Hospital (RSND – *Rumah Sakit Nasional Diponegoro*) and collections belonging to the authors' peer group in the Integrated Laboratory of Diponegoro University, Semarang. Preparation of test bacteria used a 24-hour-old bacterial suspension with an equivalent size of 1.5×10^8 CFU/ml which were inoculated using the swab method. Fungal mycelium pieces were attached to the test bacterial culture and then the petri dish was closed and incubated for 24-48 hours at 37°C temperature. Antibacterial activity was demonstrated by the presence of inhibitory zones around the *plug* of fungal association.

Fungal Cultivation

Isolate subculture was carried out using MEA media. The mycelia after growing were taken using an ose needle in the form of a small circle with a 2 mm diameter. Mycelia cuts were inoculated in a new MEA (standard) and some modified media (solid, 20 ml/petri dish), the pH was approaching neutral (approximately 5.60-6.50), and the salinity was 50-60 ‰. Furthermore, it was

incubated for 6,7,8,9 days (dark and static 24-hour conditions) at 27°C temperature. The media used were the standard MEA media, while modified media were from fish broth (M1, M2) and cassava infusion water (M3, M4). The followings are the process of making the media. MEA was made by dissolving 48 g of media in 1L of sterile sea water (composition : malt extract 30 g, peptone 3 g, agar 15 g) (Merck's protocol). M1 was made by boiling 100 g of thin slices of fish meat (*Rastrelliger* sp.) in 1L of sea water for 30 minutes and then filtered. After that, the filtrate was added with 20 g of fructose and 15 g of agar (volume of the total solution = 1 L). M2 is made as to the method for M1, but the filtrate was added with 20 g of glucose and 15 g of agar. M3 was made by boiling 100 g of thin cassava slices for 30 minutes in 1L of sea water and then filtered. The filtrate was added with 15 g of agar (volume of the total solution = 1L). M4 was made as to the method done for M3 and the filtrate was added with 3 g of peptone and 15 g of agar.

Extraction and Profiling of Secondary Metabolites

Culture media and the mycelia, after being harvested, were macerated with methanol (1: 1v/v). Then, they were partitioned using distilled water (aquadest) and ethyl acetate (1: 1v/v). After partition,

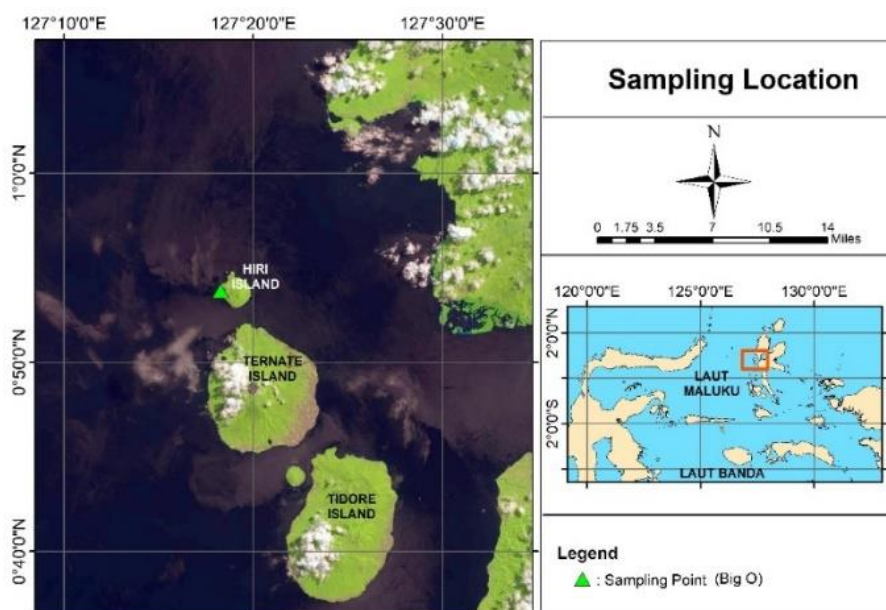


Figure1. Sampling Location of Hiri Island Waters

each solvent was evaporated using rotavapour to obtain ethyl acetate (EEs) and methanol extracts (MEs). Extract profiling used the TLC method and followed by detection of compound groups based on spot reactions to 2% vanillin-H₂SO₄ (Harborne, 1973) and 0,25% ninhydrin reagent (Sen et al., 2012). The plates were heated at 110°C for 2-3 minutes to emphasize spot sightings.

Antimicrobial Activity

The antibacterial bioassay was carried out by determining the diameter of the inhibitory zone using disc diffusion (antibacterial agar disc diffusion assay). It was conducted based on the method used by Trianto et al. (2017). Each extract in methanol solvent was tested against pathogenic bacteria using a 6 mm diameter Oxoid disc with 500 µg/disc concentration. The positive control was treated using chloramphenicol (Oxoid, 30 µg). Moreover, test bacteria were cultivated using Mueller Hinton Agar (MHA) media with (equivalent to) 1,5 x10⁸ CFU/ml initial density (REMEL McFarland Equivalence), and the bioassay was tested after the bacteria aged 24-hour-old. Inhibition zones were measured after a 24-hour incubation period at 37°C temperature.

RESULT AND DISCUSSION

The fungus isolate with code TE-BO-09.1 which was able to inhibit 3 bacteria, i.e.: *K. pneumoniae*, *B subtilis*, and *S. aureus*, but unable to inhibit *E. coli* and *P. aeruginosa* (as described in Table 1). *E. coli* and *P. aeruginosa* bacteria were included in gram-negative bacteria which are known to be resistant to various antibiotics. This condition is supported by the ability of *E. coli* to produce extended -spectrum β-Lactamases enzymes (Extended Spectrum β-Lactamases/ESBL) which can degrade certain antibiotics (β-lactam class) ((Farhat et al., 2009 ; Bimanand et al., 2017)) and can form capsules (Khan et

al., 2015), while *P. aeruginosa* can form biofilms (Alhazmi, 2015). Based on Podschun & Ullmann (1998) and Khan et al. (2015), the common triggers of malignancy from these gram-negative bacteria are the presence of endotoxin (toxin which is an integral part of cell walls of gram-negative bacteria) and adhesin (extracellular matrix and plasma protein deposits used to attach to biomaterials).

Morphological characteristics of fungus TE-BO-09.1 macroscopically and microscopically are described in Figure 3. Colonies produced by MEA cultivation had a greenish-yellow color, and when the petri dish was reversed, they looked yellowish. The conidiophore was long and the tip was round bulge and formed vesicles. The vesicles were surrounded by metula with uni- and biseriates of phialides. Besides, the tips of which were attached with many rounded form conidia. Referring to the results of previous studies, the fungal isolate used in this study was *Aspergillus flavus* (Zulkifli and Zakaria, 2017; Makhlof et al., 2019). This assumption was consistent with its molecular identification.

All fungus *Aspergillus flavus* cultivated using modified media produced greater ethylacetate extract (EE) weight than they were in the control media (MEA). The highest extract weight was achieved by the use of M4, i.e. 38.67 mg EE/20 ml media with 9-day-cultivation time, while MEA only produced 11.50 mg on 7-day-cultivation. It also occurred in methanol extract (ME), except M4 which experienced the opposite thing. M1 media produced the highest extract as much as 245.00 mg ME/20 ml media with 7-day-cultivation time, while the MEA produced 156.67mg at 8-day-cultivation. Each fungal growth media produced the highest secondary metabolites at different cultivation times. This is consistent with the study conducted by Gliseida et al. (2013), Arumugam et al. (2014), and Sibero et al.

Table 1. Antagonistic Test of Fungus TE-BO-09.1 against MDR Pathogen Bacteria

Isolate	Formation of the inhibition zones				
	<i>E. coli</i>	<i>K. pneumoniae</i>	<i>B. subtilis</i>	<i>S. aureus</i>	<i>P. aeruginosa</i>
TE-BO-09.1	-	+	+	+	-

(+) presence of inhibition zone; (-) absence of inhibition zone

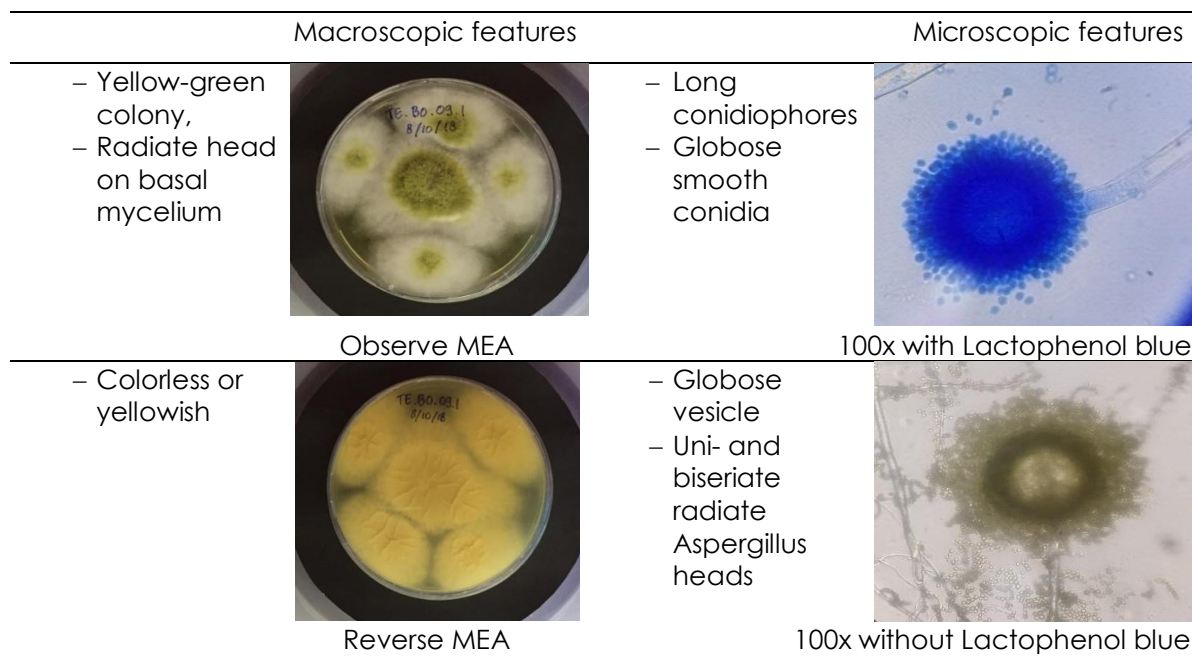


Figure 2. Morphological Characteristics of Fungus TE-BO-09.1 using MEA

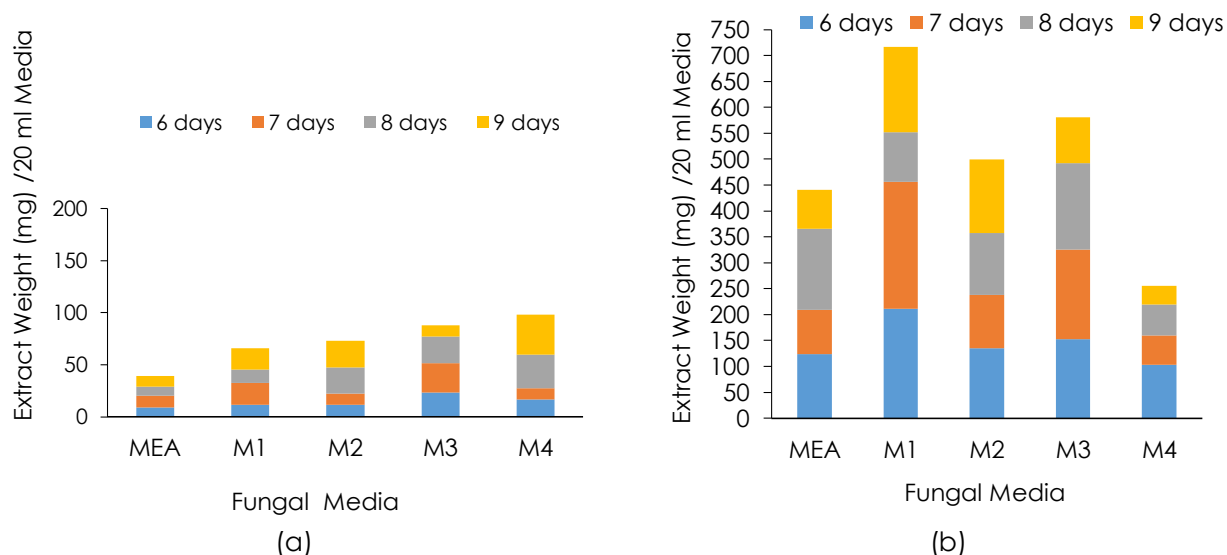


Figure 3. Weight of Fungus *Aspergillus flavus* Extracts at Different Cultivation Times (6-9 days), (a) Ethyl Acetate Extracts, (b) Methanol Extracts (Note : Standard = MEA; M1 = Fish Broth + Fructose; M2 = Fish Broth + Glucose; M3 = Cassava Infusion; M4 = Cassava Infusion + Peptone)

(2018) which stated that the production of secondary metabolites will increase as entering the stationary phase, and begins to occur on day 6 through 9 after cultivation.

Cultivation using MEA produced secondary metabolites that were naturally antibacterial through antagonistic testing (agar plug method). Nevertheless, it resulted

in negative in testing antimicrobial activity (500µg/disc). This result was probably due to its weak antibacterial characteristic, thus its active concentration was above 500 µg/disc. Antibacterial bioassays of each extract are described in Table 2 showing the strongest EE from M2 as the result of 9 days cultivation.

Table 2. Inhibition Zones of The *Aspergillus flavus* Extracts at 500 µg/disc concentration

Extract/ Cultivation Time (days)	Diameter of Inhibition Zone (in mm including the disc)		
	<i>K. pneumoniae</i>	<i>S. aureus</i>	<i>B. subtilis</i>
MEA			
Ethyl Acetate :			
6	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00
Methanol :			
6	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00
M1			
Ethyl Acetate :			
6	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00
Methanol :			
6	0.00±0.00	0.00±0.00	7.47±0.25
7	0.00±0.00	8.80±0.16	7.47±0.26
8	0.00±0.00	7.52±0.09	7.30±0.19
9	0.00±0.00	8.83±0.27	7.92±0.28
M2			
Ethyl Acetate :			
6	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	10.32±0.01	0.00±0.00
Methanol :			
6	0.00±0.00	8.04±0.50	0.00±0.00
7	0.00±0.00	8.52±0.19	0.00±0.00
8	0.00±0.00	8.83±0.50	0.00±0.00
9	0.00±0.00	9.43±0.00	0.00±0.00
M3			
Ethyl Acetate :			
6	0.00±0.00	9.32±0.00	0.00±0.00
7	0.00±0.00	9.81±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00
Methanol :			
6	7.84±0.42	0.00±0.00	8.05±0.48
7	7.65±0.00	0.00±0.00	7.31±0.15
8	7.53±0.42	0.00±0.00	7.39±0.07
9	7.58±0.33	0.00±0.00	7.14±0.45
M4			
Ethyl Acetate :			
6	0.00±0.00	0.00±0.00	0.00±0.00
7	0.00±0.00	0.00±0.00	0.00±0.00
8	0.00±0.00	0.00±0.00	0.00±0.00
9	0.00±0.00	0.00±0.00	0.00±0.00
Methanol :			
6	10.05±0.71	0.00±0.00	7.65±0.16
7	9.50±0.00	0.00±0.00	7.49±0.13
8	8.82±1.20	7.98±0.41	7.77±0.85
9	0.00±0.00	7.42±0.00	7.67±0.55
Chloramphenicol (30µg/disc)	29.70±1.32	31.37±2.05	24.43±0.93

It was able to produce a 10.32 mm inhibition zones against *S. aureus*. The largest inhibition zone of ME was achieved when fungi association was cultivated for 6 days with M4, in which it was able to produce 10.05 mm diameter inhibition zone against *K. pneumoniae*. Based on the graph in Figure 2, 9-day-cultivation time (M2; EE) and 6-day-cultivation time (M4; ME) were the periods when each media reached the highest extract weight.

The results of EEs (from all media) visualization using KLT with mobile phase n-hexane: ethylacetate (9: 1) produced several spots which positively reacted to 366 nm UV (Rf 0.44, 0.52, 0.70) and 2 % vanillin-H₂SO₄ reagent (Rf 0.78). Fluorescent blue compounds, when exposed to UV, are considered to be a group of organic compounds that possess double bonds (diene/polyene or conjugation) (Mohammed, 2018). Compounds which produce certain colored spots (varying colors) with vanillin reagents show the presence of alcohol and carbonyl functional groups (ketones, aldehydes). These compounds are included in terpenoid family, steroids, flavonoids, fatty acids/essential oils, phenolics, high molecular weight alcohols, and UV absorbing compounds. Vanillin reagents are sensitive to steroids (Harborne, 1973; Jork, 1990). Besides, the compounds which react positively to UV and vanillin are assumed to be in steroids or terpenoids categories since the results of their visualization are similar to the study done by Widyaningsih *et al.* (2018).

The results of MEs (from all media) visualization with mobile phase ethyl acetate: methanol: distilled water/aquadest (2: 2: 1) produced several spots which positively reacted to 366 nm UV (Rf 0.71, 0.88), 2% vanillin-H₂SO₄ reagent (Rf 0.39) and 0.25% ninhydrin (0.48). Harborne (1973) stated that a positive reaction with ninhydrin reagent indicates that the substance is a compound containing nitrogen (amine, peptide, or alkaloid). The results of the research conducted by Retnowati *et al.* (2018) found that compounds of β -lactam and alkaloid groups produce purple spots when sprayed with ninhydrin. Moreover, polar compounds contained in ME may be included in phenolic

groups, terpenoid derivatives, and alkaloids or other compounds containing nitrogen such as amines and peptides.

CONCLUSION

This *Aspergillus flavus* is provably able to inhibit 3 bacteria, i.e. *K. pneumoniae*, *B subtilis*, and *S. aureus*, but not able to inhibit *E. coli* and *P. aeruginosa*. Fungus cultivation used standard MEA and modified media from fish broth and cassava infusion water which produced secondary metabolites with varying quantities and antimicrobial activities. The inhibition zone of the produced extract with 500 μ g/disc concentration ranged from 7.14 to 10.32 mm. The strongest potential was shown by ethyl acetate extract from fungus cultured with fish broth M2 (9 days cultivation) which were able to produce 10.32 mm inhibition zone against *S. aureus* and methanol extracts from fungus cultured with cassava infusion water M4 (6 days cultivation), producing 10.05 mm inhibition zone against *K. pneumoniae*.

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