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Exploring Indonesian Sponge-Associated Marine *Aspergillus hortai*: Characterization of Bioactive Compounds with Potential Anti-*Escherichia coli* Properties

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ABSTRACT

Sponge-associated marine fungi are potential source for secondary metabolite compounds. The aim of this research was to investigate sponge-associated marine fungus as secondary metabolite producers against *Escherichia coli*. The fungus was isolated from Indonesian marine sponge *Stylissa* sp. and identified as *Aspergillus hortai* through a combination of morphological and molecular characteristics of ITS DNA and β-tubulin genes. The fungus was tested against *E. coli* using fungal broth and mycelial extracts. The optimized condition was achieved by fungal broth grown in corn meal broth at 6-days of shaking incubation. Fungal extract was produced using three liters of filtered fungal broth and extracted in ethyl acetate. The antibiotic activity of the extract is vulnerable to 45°C heat and basic or acidic conditions. Therefore, the extraction was done at pH 7 with evaporation at 40°C. The extract shows 7 major bands on TLC with 1 band shows activity against *E. coli* (Rf 0.81) on bioautogram. The band was observed as a yellow color and turned black in short-wave UV and did not show any fluorescence in long-wave UV. This research shows that sponge-associated marine fungi obtained from Indonesia has the potential as anti *E. coli* worth to be explored for searching new antibiotics.

1. Introduction

Seas comprise over 75% of all life on Earth and make up 70% of the planet's surface. Owing to the diverse spectrum of environmental constraints in the locations they dwell in, marine species have evolved special health-promoting qualities and bioactive substances (Lobine *et al.* 2021). Marine fungi are underutilized but potentially abundant sources of structurally varied secondary metabolites among numerous marine species. Additionally, it has been proposed that certain bioactive compounds recovered from marine invertebrates such as sponges, are produced by fungus that are associated with sponges. Recent research has revealed that marine-associated fungi are an excellent source of naturally occurring compounds with therapeutic potential. Marine fungi provided a range of natural compounds with high bioactivities because of their unique habitats, which is a significant source of novel pharmaceuticals or medicinal compounds (Doshi *et al.* 2011; Pimentel *et al.* 2011).

Antibiotics are a cornerstone of modern medicine and are used to treat infectious diseases and prevent infection in vulnerable patients, such as those undergoing surgery, or treatment for cancer. Antimicrobial resistance (AMR) is a global public health issue that threatens modern medicine in the treatment of infectious diseases (Aslam *et al.* 2018). AMR has reportedly reached a concerning stage, according to several published studies (Zaman *et al.* 2017). A recent comprehensive assessment of the global burden of antimicrobial resistance, there were

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1,27 million fatalities directly attributed to bacterial resistance to antibiotics among the over 4,95 million deaths worldwide in 2019 (Antimicrobial Resistance Collaborators 2022). The prevalence of AMR is predicted to increase, which will have an impact on mortality rates and the global economy (Founou *et al.* 2017).

Multidrug resistance in *E. coli* has emerged as a concerning problem that is increasingly reported around the world. *Escherichia coli* has a high ability to accumulate resistance genes, primarily by horizontal gene transfer, even though *E. coli* is inherently sensitive to all therapeutically relevant antimicrobial treatments. The most problematic acquisition mechanisms in *E. coli* correspond to the genes coding for extended-spectrum β-lactamases (resistance to broad-spectrum cephalosporins), carbapenemases (resistance to carbapenems), 16S rRNA methylases (pan-resistance to aminoglycosides), plasmid-mediated quinolone resistance (PMQR) genes (resistance to [fluoro]quinolones), and mcr genes (resistance to polymyxins). Additionally, *E. coli* of animal origin also frequently display resistance to additional antimicrobial substances, such as trimethoprim, fosfomycin, sulfonamides, tetracyclines, and phenicols (Poirel *et al.* 2018).

Gram-negative bacteria such as *E. coli* have the outer membrane that prevent the small molecules passively diffuse. The compounds also might be pumped out by efflux pump when passed inside the cell. Therefore, a Gram-negative antibiotic must able to cross the membranes and accumulate quickly before being pumped out to exert the desired antibacterial activity (Munoz and Hergenrother 2021). Finding new antibiotics, especially new chemical compounds with unique mechanisms of action, is a high priority to address this issue. Many significant types of antibiotics that still in use today originated from fungi, notably penicillin, which was produced by the fungus *Penicillium chrysogenum.* The search for antimicrobial compound originated from fungi has not been thorough, proven by the abundance of new chemicals that have been found in recent years (Cheng *et al.* 2012; Ishii *et al.* 2013; Wu *et al.* 2015; Myrtle *et al.* 2016). Even re-screening previously screened genera, such as *Penicillium*, has uncovered a vast array of secondary metabolites that may have been unnoticed (Nielsen *et al.* 2017).

One of the main producers of fungal secondary metabolites are from the genus of *Aspergillus.* *Aspergillus* belongs to a species complex which consist of several sections, one of them is *Aspergillus* section *Terrei*. Marine-derived isolates of *Aspergillus* section *Terrei* are well known for the production of structurally diverse and biologically active natural products. One of the economically significant species in the *Aspergillus* section *Terrei* is *A. terreus* that is involved in the synthesis of numerous secondary metabolites that are crucial to the food, fermentation, and pharmaceutical industries (Ashtekar *et al.* 2021). Although *A. terreus* is a common fungus found in tropical and subtropical areas, it may also be found in harsh climatic environment, such as severe habitats with high salinity, high alkalinity, high temperatures, as well as drought and other circumstances (Feng *et al.* 2019; Zaman *et al.* 2020). The endophytic fungus may also be isolated from a variety of hosts, including sponges, terrestrial plants, soil samples, and mangrove plants (Amr *et al.* 2023). The potential bioactivity of marine fungi especially sponge-associated marine fungi is high. However, research on sponge-associated marine fungi particularly against Gram-negative bacteria, such as *E. coli* is of high priority. Therefore, we began our research to fill this gap.

Pramuka Island is part of the Kepulauan Seribu, a group of islands in the Bay of Jakarta, Indonesia that has relatively shallow and clear waters, dominated by coral reef, seagrass and mangrove ecosystems which are important habitats for various types of tropical marine life. The potential of marine fungi of this unique habitat has not been tapped fully. Therefore, the main objective of this research was to investigate sponge-associated marine fungi as secondary metabolite producers against *E. coli* isolated from Pramuka Island, Indonesia.

2. Materials and Methods

2.1. Fungal Isolation and Antimicrobial Screening Against *Escherichia coli*

Marine sponges *Stylissa* sp. were collected by scuba diving at Pramuka Island (5°44'50,3"S, 106°36'29,8"E), Seribu Archipelago, northern part of Jakarta, Indonesia on August 2017. The sponge samples were cut and collected underwater to prevent contact of tissue with the air. The samples were stored at 4°C prior to isolation in the laboratory. The fungal isolation was conducted under aseptic conditions following the method at Fadillah *et al.* (2022). Sponge samples were rinsed three times

with sterile water then cut into 1 cm^3 small pieces of inner and outer layer of sponge pieces. The pieces planted into potato dextrose agar (PDA) containing the antibiotics chloramphenicol (500 mg/L) and fungistatic rose bengal (30 mg/L) and then incubated in 27°C for a month with three replicates. The fungi which emerge from the pieces were immediately transferred into new medium to obtain pure culture. Plugs of agar containing of mycelial growth of pure culture were tested against *E. coli* by well diffusing method. The medium was incubated in 27°C and observed for 2 days. The pure cultures which activity against *E. coli* activity were used for identification and further analysis.

2.2. Morphological and Molecular Identification of Selected Fungus

Macroscopic and microscopic morphological traits of the selected isolate such as colony characteristics, mycelia, conidiophore, phialides and conidia were observed. The selected isolate also was cultured on the top of cellophane membrane on PDA at 27°C for 5 days prior to harvesting for DNA extraction. The mycelium was harvested and ground with a sterile mortar and pestle. Fungal DNA was extracted using the cethyl trimethyl ammonium bromide (CTAB) procedure described by Sambrook *et al.* (1989). In brief, the ground mycelia in Eppendorf tube were diluted with 500 µL CTAB. Equal volume of Chloroform-Isoamyl alcohol (CI) mixture was then added to the tube. after brief mixing, the mixture was centrifuged at 10,000 rpm for 10 min at 4°C. The polar phase was transferred to a fresh Eppendorf tube and then sequentially extracted with Phenol-Chloroform-Isoamyl alcohol (PCI) mixture. Finally, the DNA was precipitated by adding absolute ethanol. The DNA pellet was then washed with ethanol 70% and resuspend in sterile nuclease free water (NFW). The DNA quality was measured by using nanodrop (MaestroGen Inc., Taiwan) with comparing the absorbance at λ 260 and 280 nm.

The extracted DNA was used as a template to amplify the fungal internal transcribed spacer 1 (ITS-1), 5.8S rDNA and internal transcribed spacer 2 (ITS-2) using ITS1 (5-TCCGTAGGTGAACCTGCGG-3) and ITS 4 (5-TCCTCCGCTTATTGATATGC-3) primers (White *et al.* 1990). Meanwhile, the amplification of the β-tubulin gene was performed using the primers Bt2a (5-GGTAACCAAATCGGTGCTGCTTTC-3) and Bt2b (5-ACCCTCAGTGTAGTGACCCTTGGC-3) (Glass and Donaldson 1995; Isshiki *et al.* 2014). The reaction mixture contained 12.5 µL PCR master mix (kappa fast 2G), 1.5 µL of 10 pmol primer (each), 3 µL of 100 ng DNA template and 6.5 µL NFW. The PCR conditions were as follows: initial denaturation (94°C for 5 min); 30 cycles of denaturation (94°C for 30 sec), primer annealing (55°C for 1 min), and elongation (72°C for 2 min), with a final elongation at 72°C for 10 min. PCR product were purified prior to sequencing analysis. Sequencing result was analyzed its homologous sequence with GenBank and Mycobank database by using the program Basic Local Alignment Search Tool (BLAST) at http://blast.ncbi.nlm.nih.gov. The phylogenetic trees of *Aspergillus* section *Terrei* were constructed by using the MEGA X program with *Aspergillus flavipes* CBS 260.73 (MH860679) and *Aspergillus neoflavipes* NRRL 5504 (EU014084) used as an outgroup for ITS rDNA and β-tubulin phylogenetic tree respectively.

2.3. Medium Selection for Anti *E. coli* Assay

Five different liquid media were used, namely malt extract broth (ME), corn meal broth (CM), Saboraud liquid medium (SL), potato dextrose broth (PDB), and PDB with sea water (PDB-SW). Plugs of agar containing of mycelial growth of selected fungi were cut and transferred into five different media separately. Erlenmeyer flasks (250 ml) containing 50 ml medium were incubated at 27°C at 150 rpm shaking speed for 14 days. The cultures from every medium were harvested every 2 days and then the mycelium was separated from broth by centrifugation at 6,000 rpm for 10 minutes. The mycelium was extracted with double the volume of methanol for 4 hours prior to subjected for antimicrobial assay. The antimicrobial activity of the supernatant (broth) as well as the mycelial extract of the fungi were tested in agar diffusion assay against the *E. coli*. The assay was done by testing 50 µL of samples in 6 mm diameter of well.

2.4. Optimization and Characterization of Antimicrobial Properties on Fungal Broth

Antimicrobial compound in fungal broth are necessary to characterized to avoid damage to their chemical structures during purification. The medium that shows highest antimicrobial activity and harvested at the optimal incubation time was used for this characterization. The optimization was done according to Hasaneen *et al.* (2022) with some

modification. The broth was tested against *E. coli* after incubation under different conditions, such as adjusted in different pH (4, 7, and 9), incubated at 45°C for 4 hours (tested every hour) and compound solubility test by extraction in various solvent such as in hexane, ethyl acetate and butanol to obtain the optimum condition for isolation of metabolites. The assay was done as described above (Section 2.3).

2.5. Production and Extraction of Fungal Compound on a Larger Scale

The best medium supporting the production of the antimicrobial activity from the previous assay (Section 2.3 and 2.4) were used for cultivating the fungal compound. Plugs of agar supporting mycelium were transferred to 3 L of medium. Flasks were incubated at 150 rpm at 27°C for 6 days. The culture was tested again for antimicrobial activity to ensure the antimicrobial compound was produced. The assay was done according to the optimization in Section 2.4. The culture broth was separated from mycelia using vacuum filter then the broth was extracted twice with ethyl acetate (1:0,5) and then dried by rotary evaporation to give a yellowish-brown crude extract.

2.6. Isolation of Active Compound

The crude extract from the 3 L of large-scale production was subjected to TLC with 10 × 10 cm^2 silica gel plate (TLC silica gel 60 F254) and ethyl acetate as solvent and bioautogram to estimate the polarity of the active compound. The crude extract was fractionated by using column chromatography with 300 ml silica gel 230-400 mesh as resin. The crude extract was loaded in a small volume of chloroform directly on the top of column when the resin was set on column and provided no air bubbles. The eluent used in this column chromatography as follows, chloroform-ethyl acetate (gradient 9:1, 8:2, 7:3, 5:5, 3:7, 0:1) then ethyl acetate-methanol (gradient 95:5 and 90:10) each were added at 200 ml into the column carefully. The fractions were collected to a small flask for every 50 ml of flow-through. The collection was stopped when the last flask shows no observed band when on TLC. A bioautogram test was conducted for every odd-numbered flask harvested from column chromatography. The bands of compound which shows positive antimicrobial activity were marked and the fractions showing similar bands were collected into one flask each. The flasks containing active compound were dried and weighed prior to further purification by separation with preparative TLC method.

The extract fraction then subjected to preparative TLC (TLC-p) with silica gel resin on glass plate. Ethyl acetate was used as eluent for fraction with moderate retention factor value (RF) and ethyl acetate: methanol (8:2) was used for high RF fraction. The dried fractions were diluted to 1-2 ml of ethyl acetate then spotted 1 cm from bottom part of 20 × 20 TLC-p plate. The bands were visualized under UV light and marked with a pencil. The bands were harvested into glass tubes and then eluted with ethyl acetate: methanol (9:1). Collected fraction were separated from the silica sediment by centrifugation. The silica sediment then washed with ethyl acetate until shows no bioactive compound left on sediment by using TLC silica plate. The solution of fraction obtained then dried with liquid nitrogen and weighed.

3. Results

3.1. Fungal Isolation and Antimicrobial Screening Against *Escherichia coli*

The depth of collected sponge was of approximately 10 meters below sea level. The environmental parameter was 28°C with 7.7 pH and clearly visible. Based on sponge classification, the sponge was classified to *Stylissa* genera (Figure 1). A total of 23 fungal isolates obtained from the sponge with only one isolate shows positive activity against *E. coli*. The isolate then was selected for further analysis and stored at IPB culture collection with the code IPBCC.19.1497.

3.2. Morphological and Molecular Analysis of Selected Fungi

The selected marine fungus showed anti *E. coli* activity was isolated from inner layer of marine sponges *Stylissa* sp. and identified as *Aspergillus* sp. IPBCC.19.1497 based on the identification employing morphological characteristics (Figure 2). The fungal characteristics were as follows. Fungal colonies grown on PDA (Oxoid) at 27°C were 67.4±1.8 mm in diameters after seven days of incubation. The colonies were consisted of a dark brown centre with hyaline-white felt and slightly floccose of young mycelium at the edges of the colonies. Reverse colony were in bright yellow. Somatic hyphae were 2.0–4.0 µm wide, hyaline. Conidiophores were 3.0–9.0 µm in diameter.

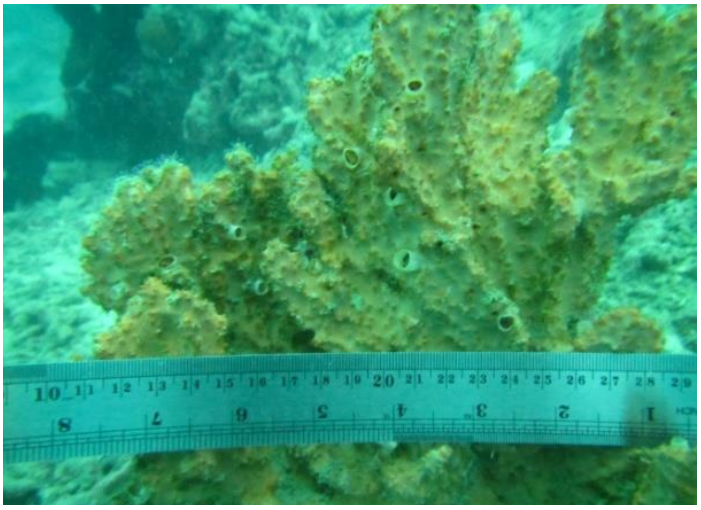

Figure 1. *Stylissa* sp. marine sponge collected from Pramuka Island, Indonesia

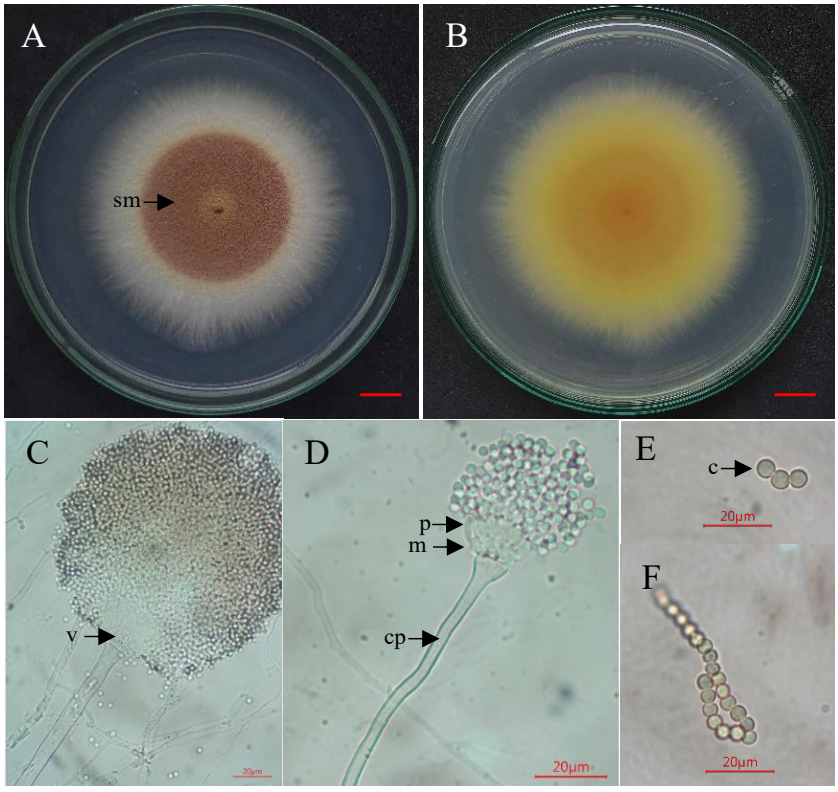

Figure 2. Colony and microscopic structures of *Aspergillus* sp. IPBCC.19.1497 grown on PDA, the microscopic structures observed at 7 days after inoculation. (A-B) Top and underside view of *Aspergillus* sp. IPBCC.19.1497 sporulating colonies, (C-D) Anamorphic structures of *Aspergillus* sp. IPBCC.19.1497, (E-F) conidia of *Aspergillus* sp. IPBCC.19.1497, (sm) spore mass, (v) vesicle, (p) phialide, (m) metula, (cp) conidiophore, (c) conidia. Scale bars are 1 cm (A-B) and 20 µm (C-F)

Vesicles were biseriate, globose to subglobose 7.0–17.0 µm wide. Conidia were globose, dark brown en masse, 2.7–3.3 µm in diameter.

Further identification showed that with BLAST analysis of ITS rDNA *Aspergillus* sp. IPBCC.19.1497 was homologous with *A. hortai* strain CBS 124230 (KP987087) with 100% coverage of sequence shows 99% similar identity, while β-tubulin gene showed *Aspergillus* sp. IPBCC.19.1497 was homologous with *A. hortai* strain NRRL 274 (FJ491706) (Table 1).

Phylogenetic tree of *Aspergillus* sp. IPBCC.19.1497 were constructed with Maximum Likelihood method based on the Tamura 3-parameter model for ITS rDNA and Kimura 2-parameter model for β-tubulin gene. The strength of the branches of phylogenetic trees were tested 1,000 replicates as bootstrap by using MEGA X program (Figure 3 and 4). The phylogenetic tree of ITS rDNA shows that *Aspergillus* sp. IPBCC.19.1497 closely nested with *A. terreus, A. neoafricanus, A hortai* and other *Aspergillus* species in the section *Terrei*. The phylogenetic tree of β-tubulin gene shows that *Aspergillus* sp. IPBCC.19.1497 was closely related to *A. hortai* IBT 26384 and distinctly separated with other species among the species in *Aspergillus* section *Terrei*. The result of β-tubulin phylogenetic tree was in line with BLAST analysis. Therefore, the fungus will be further called as *A. hortai* IPBCC.19.1497.

Table 1. BLASTn result of *Aspergillus* sp. IPBCC.19.1497

IPB culture collection code	BLASTn result				
	Gene region	Homologous result	Query cover	Identity	Accession number
IPBCC.19.1497	ITS rDNA	*Aspergillus hortai* strain CBS 124230	100	99	KP987087
	β-tubulin	*Aspergillus hortai* strain NRRL 274	100	100	FJ491706

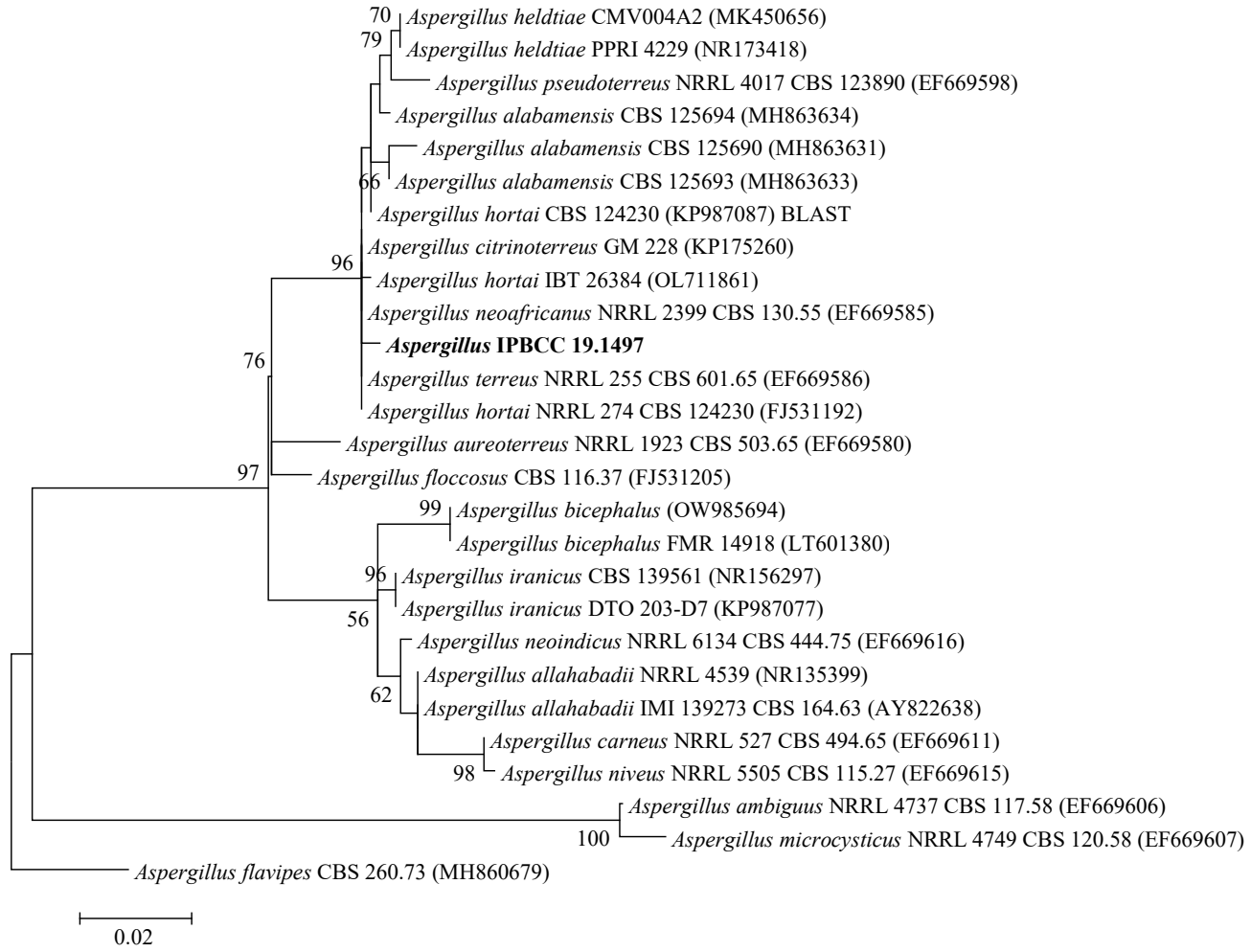

Figure 3. Phylogenetic tree of ITS rDNA gene of *Aspergillus* section *Terrei* obtained by maximum likelihood analysis. Phylogenetic analysis was performed on the Tamura 3-parameter model with invariant rates among sites. The tree was constructed with *A. flavipes* as outgroup. Bootstrap values are indicated at the nodes

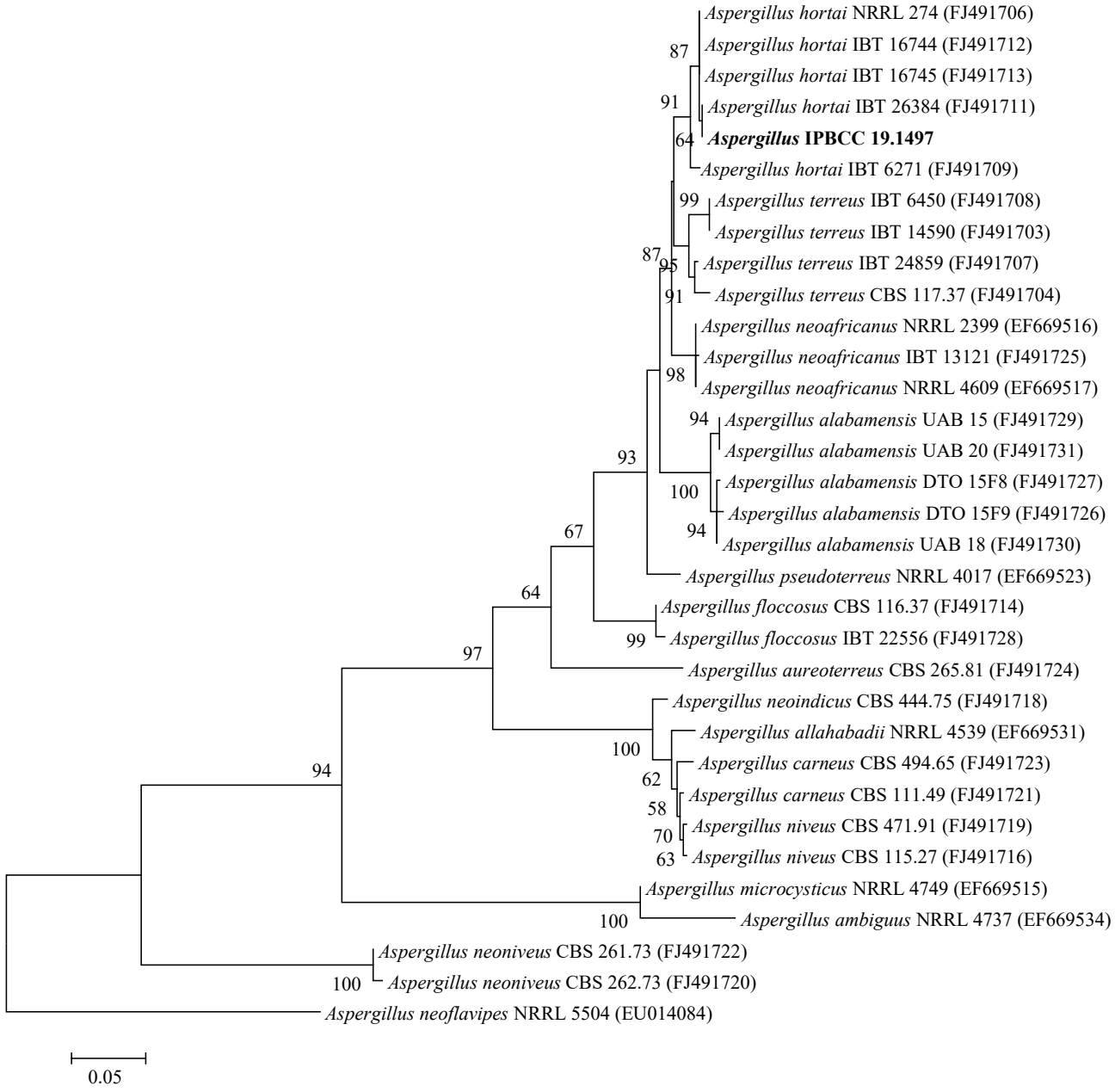

Figure 4. Phylogenetic tree of β-tubulin gene of *Aspergillus* section *Terrei* obtained by maximum likelihood analysis. Phylogenetic analysis was performed on the Kimura 2-parameter model with gamma distribution among sites. The tree was constructed with *A. neoflavipes* as outgroup. Bootstrap values are indicated at the nodes

3.3. Best Medium Selection for Production of Anti *Escherichia coli* Assay

The activity of *A. hortai* IPBCC.19.1497 against *E. coli* was tested using fungal broth and mycelial extracts. The assay shows that the fungus *A. hortai* IPBCC.19.1497 produced extracellular antimicrobial compound which was observed in fungal broth when tested against *E. coli* while mycelial extract in methanol (intracellular) did not shown significant activity against *E. coli* (Figure 4). Five different media were used and compared in this study, namely corn meal broth (CM), malt extract broth (ME), Saboraud liquid medium (SL), potato dextrose broth (PDB), and PDB with sea water (PDB-SW). All five media showed slightly different growth rates due to the differences between nutrients in each medium. The antimicrobial activities of each medium were begin observed from four days of incubation and the activity peaked at six days of incubation in every medium. The decrease of antimicrobial activities was observed starting

from day 8 and sharply decreased until 14 days of incubation (Figure 5). The antimicrobial assay using five different medium shows different strength of antimicrobial activity with the optimal condition to obtain the best antimicrobial compound are by using corn meal broth (CM) for 6 days of incubation time. In this study, the fungal broth of CM at 6 days of incubation showed the highest inhibition activity against *E. coli* with 24.4±0.245 mm in diameter of inhibition.

3.4. Optimization and Characterization on Anti *Escherichia coli* Properties of Fungal Broth

Fungal broth from CM medium incubated for 6 days were used in optimization against *E. coli.* The broth was subjected at pH treatment (4, 7, and 9), 45°C incubation treatment and tested its solubility in organic solvent. The bioactivity of *A. hortai* IPBCC.19.1497 broth showed a decreasing activity at acidic and basic condition (Figure 6). Therefore, to maintain the activity, the broth or extract obtained from this fungus must not be exposed to acid or base materials. The bioactivity of *A. hortai* IPBCC.19.1497 broth also showed a significant decrease of activity when exposed in 45°C heat for a long time (Figure 7). Therefore, to obtain the bioactive compound, the incubation and the extraction process must not be done in higher temperature (higher than 45°C). The bioactive compound of *A. hortai* IPBCC.19.1497 shows a good solubility in ethyl acetate and obtain a better extraction when extracted twice, shown by

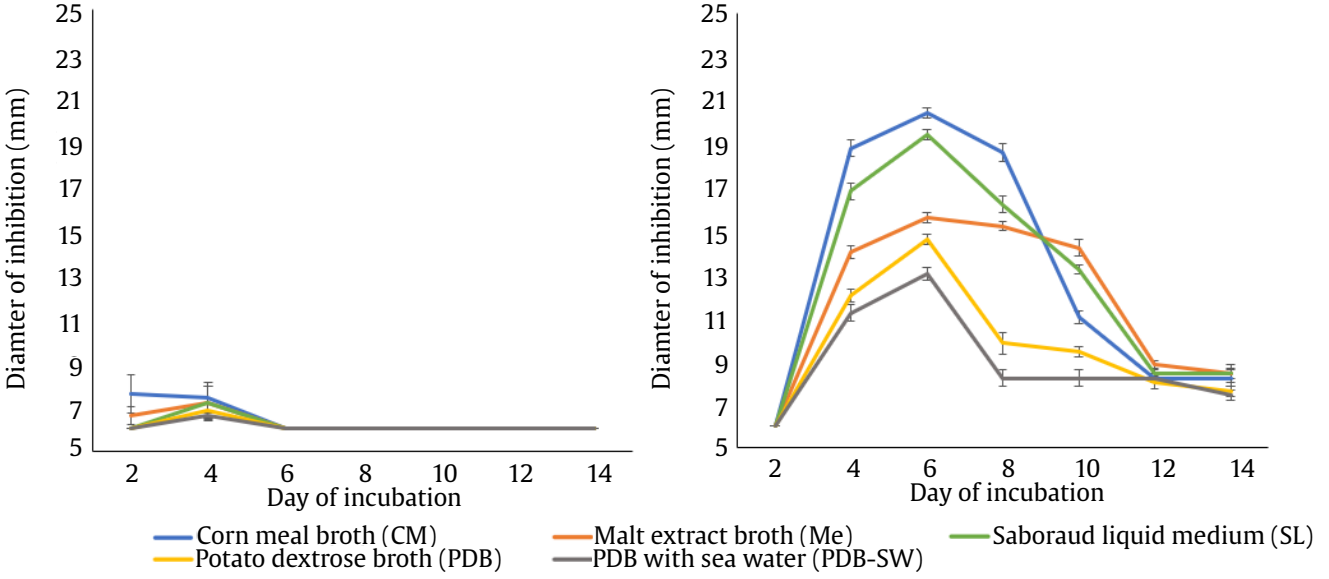

Figure 5. The activity of *A. hortai* IPBCC.19.1497 against *E. coli* on several broth medium; (left) mycelia, (right) broth. The diameter of inhibition was measured in five replicates and the bar indicated the error standard of the measurements

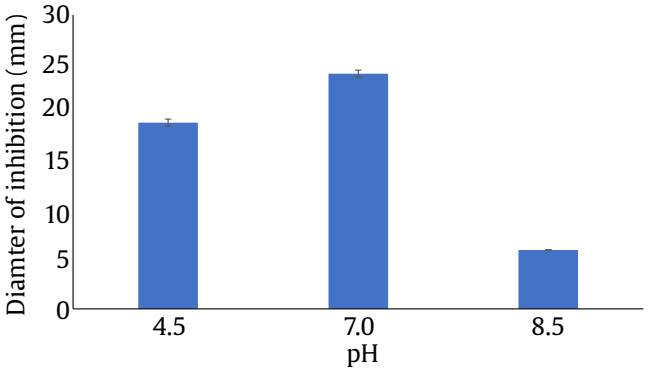

Figure 6. Effect of *A. hortai* IPBCC.19.1497 broth against *E. coli* when subjected at pH treatment. The diameter of inhibition was measured in five replicates and the bar indicated the error standard of the measurements

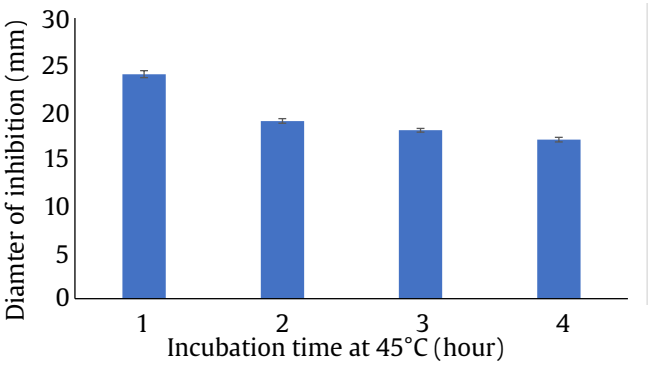

Figure 7. Effect of *A. hortai* IPBCC.19.1497 broth against *E. coli* when subjected at 45°C incubation. The diameter of inhibition was measured in five replicates and the bar indicated the error standard of the measurements

a significant decrease of spent activity against *E. coli* (Figure 8). Therefore, the fungal bioactive compound could be extracted with ethyl acetate with multiple steps of extraction to obtain the maximum result of the compound.

3.5. Extraction, Isolation and Characterization of Fungal Active Compound

The 3 L broth of *A. hortai* IPBCC.19.1497 was extracted according to preliminary studies of the bioactive compound. The extraction was done by hand-shaken mixing with ethyl acetate as solvent twice. The extract then evaporated by rotary evaporation at 40°C, in small batches to reduce the heating time, to give a 200 ml yellowish-brown crude extract. The extract shows 7 major bands on TLC with ethyl acetate (rf: 0,31; 0,57; 0,69; 0,79; 0,81; 0,88; 0,94). Bioautogram profile from 7 major band shows one band (rf 0.81) had activity against *E. coli* (Figure 9). The bioautogram of the flow through shows the active bands contained in flasks 3-9 (rf 0,81). The flow through then collected in one flask then dried prior to be used in TLC-preparative (TLC-p). The active compounds were then spotted in TLC-p plate and then collected into the test tube. Collected fraction then diluted with ethyl acetate and

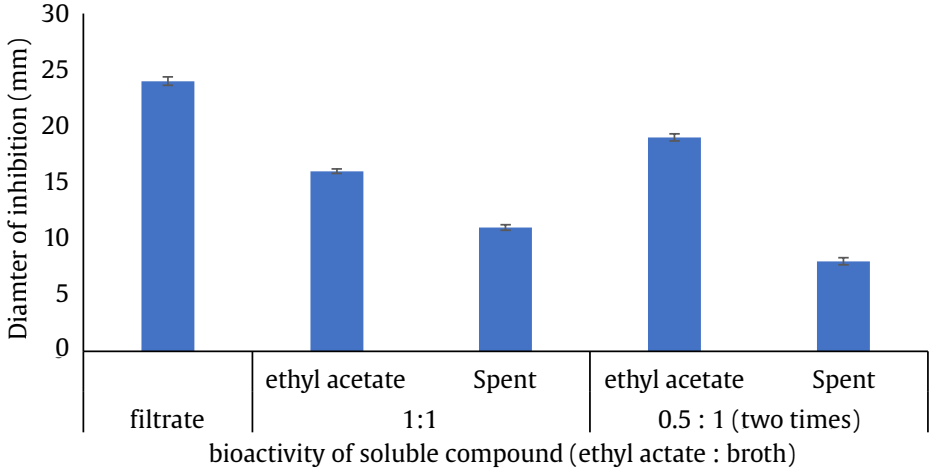

Figure 8. Solubility of *A. hortai* IPBCC.19.1497 bioactive compound against *E. coli* when extracted with ethyl acetate. The diameter of inhibition was measured in five replicates and the bar indicated the error standard of the measurements

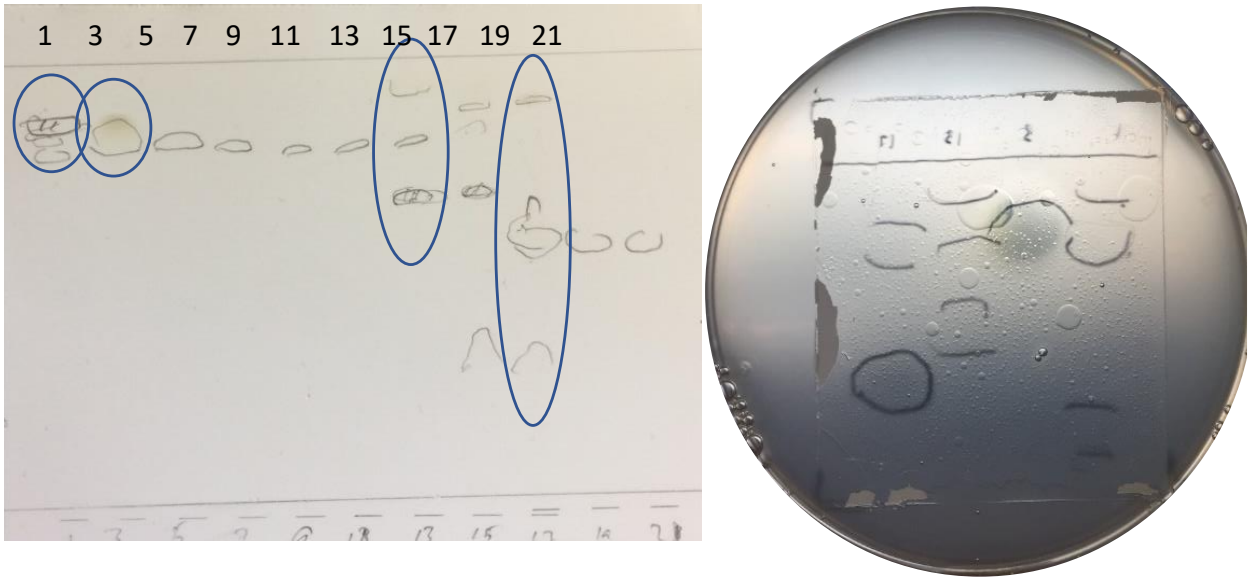

Figure 9. The TLC profile of odd-numbered flask of flow through (left), The flask number 1, 3, 13, and 17, marked with circle, are contains 7 major band and were selected for bioautogram. The flask number 3 showed inhibition against *E. coli* (right)

shaken prior to separate from silica sediment. The solution of fraction then dried with liquid nitrogen and weighed. The amount obtained from TLC-p were a total of 11.7 mg. Purified fraction obtained from TLC-p in small quantities and observed containing a mixture of compounds. Therefore, the fraction obtained was needed to purified further and was not enough to continue in the elucidation process.

4. Discussion

The marine fungus that showed anti *E. coli* activity was isolated from inner layer of *Stylissa* sp. Based on the identification employing morphological characteristics, the fungus identified as *Aspergillus* sp. IPBCC.19.1497. Further identification employing molecular identification of ITS rDNA with BLAST analysis showed that, *Aspergillus* sp. IPBCC.19.1497 is homologous with *Aspergillus hortai* strain CBS 124230 (KP987087) with 100% coverage of the sequence and 99% similar identity. Based on phylogenetic analysis, the fungus was closely related to *Aspergillus terreus* NRRL 255 CBS 601.65 (EF669586). The fungal identification based on ITS rDNA barcoding is not distinctly separated among *Aspergillus* species. Therefore, a polyphasic approach in molecular identification is needed in order to identify the fungus. A polyphasic approach using sequence analysis of parts of the β-tubulin genes alongside the ITS region was required to identify among *Aspergillus* species in the section *Terrei* (Samson *et al.* 2011, 2014). The BLAST analysis of the fungus with the β-tubulin gene showed that *Aspergillus* sp. IPBCC.19.1497 was homologous with *A. hortai* strain NRRL 274 (FJ491706) with 100% coverage of the sequence and 100% similar identity. Based on the phylogenetic analysis of the β-tubulin gene, the species of *Aspergillus* in the section *Terrei* are distinctly separated and the *Aspergillus* sp. IPBCC.19.1497 was closely related to *A. hortai* IBT 26384 (Figure 4). Therefore, the fungus was identified as *Aspergillus hortai* IPBCC.19.1497.

Although *Aspergillus* section *Terrei* is a common fungus found in tropical and subtropical areas, it may also be found in harsh climatic environments, such as severe habitats with high salinity, high alkalinity, high temperatures, as well as drought and other circumstances (Feng *et al.* 2019; Zaman *et al.* 2020). The fungus may also be isolated as endophytic fungi from various hosts, including sponges, terrestrial plants, mangrove plants, soil samples, and mangrove plants (Amr *et al.* 2023). *Aspergillus hortai* IPBCC.19.1497 is categorized as a facultative marine fungus due its ability to grow on media without seawater. It is possible that the fungus was carried from a terrestrial habitat into the sea and then associated with sponges.

The antimicrobial activity of *A. hortai* IPBCC.19.1497 was further tested against the Gram-negative bacteria *E. coli*. The assay showed that *A. hortai* IPBCC.19.1497 had antimicrobial activity against *E. coli* by producing extracellular metabolites (Figure 4). These metabolites are produced optimally on CM at six days of incubation. The Antimicrobial biosynthesis of microorganisms depends on the culture conditions (Singh *et al.* 2017). Altering the nutrient sources (carbon, nitrogen, phosphorus, minerals, and trace elements) and environmental factors (pH, temperature, time, agitation) can greatly affect the production of secondary metabolites. Therefore, designing a suitable fermenting medium holds importance for the mass production of secondary metabolites (Sharma *et al.* 2020).

The preliminary studies of the metabolites produced by *A. hortai* IPBCC.19.1497 are important to obtain the optimum condition during the extraction and maintain the condition of antimicrobial activities by avoiding environmental changes such as temperature and pH. In this study, the antimicrobial activities of metabolites showed decreasing activities in the presence of heat (45°C) and changes in pH. The solubility of metabolites was also studied. The bioactive metabolites are greatly soluble in ethyl acetate compared to *n*-hexane and n-butanol (data not shown). Therefore, the bioactive metabolites are considered semi-polar compounds. The extraction was carried out by producing three liters of fungal broth and then extracted with ethyl acetate (1:0.5) twice by hand shaking for 1 hour. The extract was then evaporated at 40–41°C with a rotary evaporator since the compound showed decreasing activities at 45°C. The fraction band of the rf 0.81 (further called Fraction A) was yellow in color and showed a dark spot/band in short-wave UV illumination but no fluorescence in long-wave UV illumination. As much as 11.7 mg of Fraction A was obtained from 3 liters of fungal broth. The small quantities and a mixture of compounds observed in the Fraction A resulted in ending the step for elucidation, and the fraction was then collected for further studies.

The sponges as the most primitive sea invertebrates, contain a variety of microbes that are vital sources of powerful natural compounds. *Aspergillus* derived from sponge was the source of antimicrobial compounds. Most of the compounds had a wide antimicrobial spectrum against a variety of bacteria and fungi. In a broad sense, it has been reported that fifteen antimicrobial compounds were found in seven *Aspergillus* fungi strains derived from sponge (Li *et al.* 2023). *Aspergillus hortai* are known to produce several metabolites such as asterric acid, 6-methyl-4,5,7-trihydroxyphthalide, cytochalasin, asterric acid derivative, aspergillamide, butyrolactones, dicitrinin, erdins, geodin, terreic acid, terrein, and terrequinone (Barros Correia *et al.* 2020). Nevertheless, none of them was reported as antimicrobial compound. However, several similar metabolites from *Aspergillus* section *Terrei* are reported producing several antimicrobial compounds, such as terrein from *Aspergillus terreus* has potent activity against *Cryptococcus neoformans* (Cadelis *et al.* 2022). Terreic acid and butyrolactone I from *Aspergillus terreus* var. terreus shows activity against *Erwinia carotovora, Bacillus subtilis, B. brevis, Micrococcus luteus* and *Enterobacter dissolvens* (Cazar *et al.* 2005). (22E,24R)-stigmasta-5,7,22-trien-3-β-ol from endophytic *A. terreus* actively against MRSA (Ibrahim *et al.* 2015). Other antimicrobial compounds are reported from *Aspergillus* section *Terrei*, such as *A. alabamensis* produced two diketomorpholine derivatives and a highly conjugated ergostane-type steroid exhibited inhibitions against *E. coli*, *M. luteus, E. ictaluri*, and *V. alginolyticus* (Yang *et al.* 2018), *A. allahabadii* produced allahabadolactone B and (22E)-5α,8αepidioxyergosta-6,22-dien-3β-ol against *B. cereus* (Sadorn *et al.* 2016), *A. carneus* produced potential antimicrobial compound such as carneamides A-C, carnequinazolines A-C, carnemycin A-B and a drimane sesquiterpenoid (Zhuravleva *et al.* 2012), *A. niveus* produced a weak antibacterial aspochalamins A-D (Gebhardt *et al.* 2004), and *A. microcysticus* produced a well-known antimicrobial compound, asposterol (Heberle *et al.* 1974). Terrestrial *Aspergillus terreus* also had been reported producing antibacterial compound such as, 2-methyl-3-methylene-cyclopentanecarboxaldehyde; 2,4-di-tert-butylphenol; 1,3,4,6,7,8a-hexahydro-2,5,5-trimethyl-2H-2,4a-ethanonaphthalen-8(5H)-one; dibutyl phthalate; 4,5-dimethoxy-2-ethoxy-1-(2-propenyl) benzene; fluorenone oxime; and n-tridecyl ester trifluoroacetic acid (Oleru *et al.* 2021). However, all compounds mentioned above are mostly obtained from terrestrial *Aspergillus* section *Terrei*. There are no reports on antimicrobes produced by *Aspergillus hortai* as a member of *Aspergillus* section Terrei particularly obtained from marine environment.

In conclusion, the Indonesian marine fungus associated with *Stylissa* sp. sponge identified as *Aspergillus hortai* IPBCC.19.1497 produced one fraction of potential secondary metabolites with antimicrobial activity against *E. coli*. This is the first report showing marine *Aspergillus hortai* produces secondary metabolite against *E. coli*. The fungal compounds were extracted from the broth culture using ethyl acetate. Indeed, further analysis is needed to identify the compound. However, this research shows that sponge-associated marine fungi obtained from Indonesia have the potential as anti-microbes, particularly against *E. coli*.

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