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ANTIBACTERIAL ACTIVITY PROFILE OF MANGROVE ENDOPHYTIC FUNGI ISOLATED FROM BERAU REGENCY, INDONESIA

Profil Aktivitas Antibakteri Isolat Kapang Endofit Mangrove dari Kabupaten Berau, Indonesia

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ABSTRACT
Mangrove endophytic fungi have the potential to produce secondary metabolites with antibacterial properties. This study aimed to obtain endophytic mold isolates associated with mangrove plants and their antimicrobial activity. Seventeen endophytic molds were isolated from 7 mangrove species originating from Berau, East Kalimantan, Indonesia. Three extracts from the mold isolates OJ-B, OJ-Bu, and 6Xg-Bh showed the highest antibacterial activity with an inhibition zone of 9.63–25.4 mm and with MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values of around 0.625–5 and 2.5–10 mg mL^{-1}, respectively. LC-MS (liquid chromatography-mass spectrometry) analysis identified several compounds with antibacterial potential, namely beauvericin, choline, nicotinic acid, pyridoxine, quinoline, cyclo (phenylalanyl-prolyl), fusarin c, oleamide and borrelidin. The 3 species were molecularly identified as Diaporthe phaseolorum, Fusarium proliferatum *and* Phomopsis *sp. Thus, the endophytic molds from several of the mangrove species produced bioactive compounds as antibacterial.*

Keywords: *antibacterial, chemical compound, endophytic fungi, mangrove, secondary metabolites*

ABSTRAK
Kapang endofit mangrove berpotensi menghasilkan metabolit sekunder yang bersifat antibakteri. Penelitian ini bertujuan mendapatkan isolat kapang endofit yang berasosiasi dengan tanaman mangrove beserta aktivitas antimikrobanya. Tujuh belas kapang endofit diisolasi dari 7 spesies mangrove asal Berau, Kalimantan Timur, Indonesia. Tiga ekstrak dari isolat kapang OJ-B, OJ-Bu, dan 6Xg-Bh memiliki aktivitas antibakteri tertinggi dengan zona hambat 9.63–25.4 mm dan dengan nilai MIC dan MBC masing-masing sekitar 0.625-5 dan 2.5–10 mg mL^{-1}. Analisis LC-MS menunjukkan beberapa senyawa dengan potensi antibakteri yaitu beauvericin, choline, nicotinic acid, pyridoxine, quinoline, cyclo (phenylalanyl-prolyl), fusarin c, oleamide dan borrelidin. Uji molekuler menunjukkan 3 spesies terindentifikasi sebagai *Diaporthe phaseolorum, Fusarium proliferatum* dan *Phomopsis* sp. Hasil menunjukkan kapang endofit dari beberapa spesies mangrove tersebut mampu menghasilkan senyawa bioaktif sebagai antibakteri.

Kata Kunci: antibakteri, kapang endofit, mangrove, metabolit sekunder, senyawa kimia

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INTRODUCTION

Mangroves are plants that grow on the beach and are widely used in traditional medicine. Indigenous people use mangroves to treat fever, asthma, skin diseases, rheumatism, and minor injuries from insect bites. The part of the mangrove plant used includes fruit, skin, leaves, stems, and roots (Bibi et al. 2019). The natural bioactive compounds in mangroves, such as triterpenoids, alkaloids, phenolics, lignans, flavonoids, steroids, and terpenoids, are widely used as antimicrobial, anticancer, and antioxidants (Prihanto et al. 2018). However, the utilization of mangrove plants in producing bioactive compounds is time-consuming and needs a large amount of biomass. Mangrove excessive use will also interfere with the preservation of nature. An alternative that can be done is by utilizing endophytic microbes associated with mangrove plants as their host.

Endophytic microbes have been known capable of producing the same bioactive compounds as their hosts. So, to obtain these bioactive compounds, it is unnecessary to extract large plant parts, and sustainable mangroves can be maintained. The advantages of using endophytic microbes as producers of secondary metabolites are that microbes are culturable in bioreactors as required, bioactive compounds can be produced continuously, and the production process is relatively easy to do by microbial culture media conditioning (Kaul et al. 2013). Several studies on endophytic mangrove as antibacterial have been reported. For example, *Acanthus ilicifolius* mangrove in China's Hainan Province was shown to inhibit bacterial pathogen *Bacillus subtilis* and *Pseudomonas aeruginosa* (Cai et al. 2017a).

Antibacterial is a typical compound used in the food industry for preservation. Antibacterial is also widely used to prevent or reduce the spread of foodborne diseases. For instance, food preservation in preventing the growth of spoilage bacteria that live on food can be inhibited by adding antibacterial extract (Hintz et al. 2015). Pathogenic microbes cause food spoilage, reduce the suitability for consumption, and increase the risk of disease and toxins caused by the activity of biological agents (Septiana and Simanjutak 2016).

Mangrove plants probably contain several endophytic microbes in their tissues that can produce secondary metabolites compounds. Endophytic microbes from mangroves are also part of the second biggest marine microbe group (Zhou et al. 2018). This kind of endophytic microbes, especially endophytic fungi, has an essential role in supporting the ecosystem of mangroves during their cycles. It has been proven that endophytic fungi are the source of natural compounds with high biological activity and structural diversity (Vijaya 2017). A study conducted by Cai et al. (2017b) isolated endophytic microbes from the mangrove *Acanthus ilicifolius* from the South China Sea. A strain of *Talaromyces stipitatus* SK-4 was collected from the leaves of *A. ilicifolius,* and its crude extract showed antibacterial activities against *B. subtilis* and *Staphylococcus aureus*. Further chemical investigation of the crude extract resulted in seven depsidone derivatives. Research exploring antibacterial compounds from endophytes grown in Indonesian mangroves is limited. Thus, the purpose of this study was to obtain the endophytic fungi isolates associated with mangrove plants and their antimicrobial activity.

MATERIALS AND METHODS

Location and time

This research was carried out from August 2020 to February 2021 at the SEAMEO BIOTROP (Southeast Asian Regional Center for Tropical Biology) Biotechnology Laboratory, Bogor, West Java, and the Metabolomics Laboratory, Advanced Research Laboratory Unit, IPB University, Bogor, West Java.

Materials

The main materials used in this study were roots, stems, leaves, fruit, and flowers of several types of mangroves (namely *Rhizophora mucronate*, *R. apiculate, Sonneratia alba, S. caseolaris, Avicennia marina, Xylocarpus granatum, Nypa frutican,* and *A. ilicifolius*) obtained from the mangroves area in the Berau Regency, East Kalimantan. Materials and chemicals used in the culture and DNA isolation of the fungi included Potato Dextrose Agar (PDA) (Himedia), agar bacto (Himedia), Mueller

Hinton Broth (MHB) (Himedia), Tryptone Soy Broth (TSB) (Oxoid), distilled water, chloramphenicol, NaOCl, ethyl acetate (EtOAc) (Merck), 70% ethanol, primers ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3'), as well as 96-well microtitration plate. Total DNA isolation from fungal mycelia was carried out using the DNeasy kit (Qiagen, Germany). Amplification of target genes was performed using Gotaq Master Mix PCR (Promega, USA), agarose gel, Buffer TE, GoTagGreen, PCR master mix (Promega), and ZymocleanTM DNA Recovery Kit (Zymo Research). Bacteria for the fungal antibacterial test, namely *S. aureus* ATCC 25922, *Escherichia coli* ATCC 25922, *B. cereus* ATCC 10876 and *Salmonella typhimurium* ATCC 14028 were of the collections of the Food Microbiology Laboratory, PAU, IPB University.

Isolation of endophytic fungi

The samples used in this study were fruits, flowers, leaves, stems, and roots of several species of mangroves from Berau Regency, East Kalimantan, Indonesia, namely *R. mucronata, R. apiculata, S. alba, S. caseolaris, A. marina, X. granatum, N. frutican,* and *A. ilicifolius* (Figure 1). Cultivation of endophytic fungi began with pre-treatment of the samples. First, the mangrove samples were sterilized by removing contaminant microbes from the surface of the samples. Washing of the samples was carried out under running water for 10 minutes, followed by cutting into 1-cm long pieces. The sample pieces were then successively immersed in 75% ethanol for 1 minute, 2% NaOCl (sodium hypochlorite) for 3 minutes, and 75% ethanol for 30 seconds before cultivation on PDA (Potato Dextrose Agar) media at 28 ºC for 7–14 days. Morphologically different colonies were transferred to different PDA media, incubated for 7 days at room temperature.

Fermentation and extraction

Fermentation was carried out by inoculating a loopful of the fungal mycelia grown on the PDA plate into 250-mL Erlenmeyer flasks, each containing 100 mL PDB media. The flasks were incubated at 28 ºC, circa 130 rpm, for 14 days.

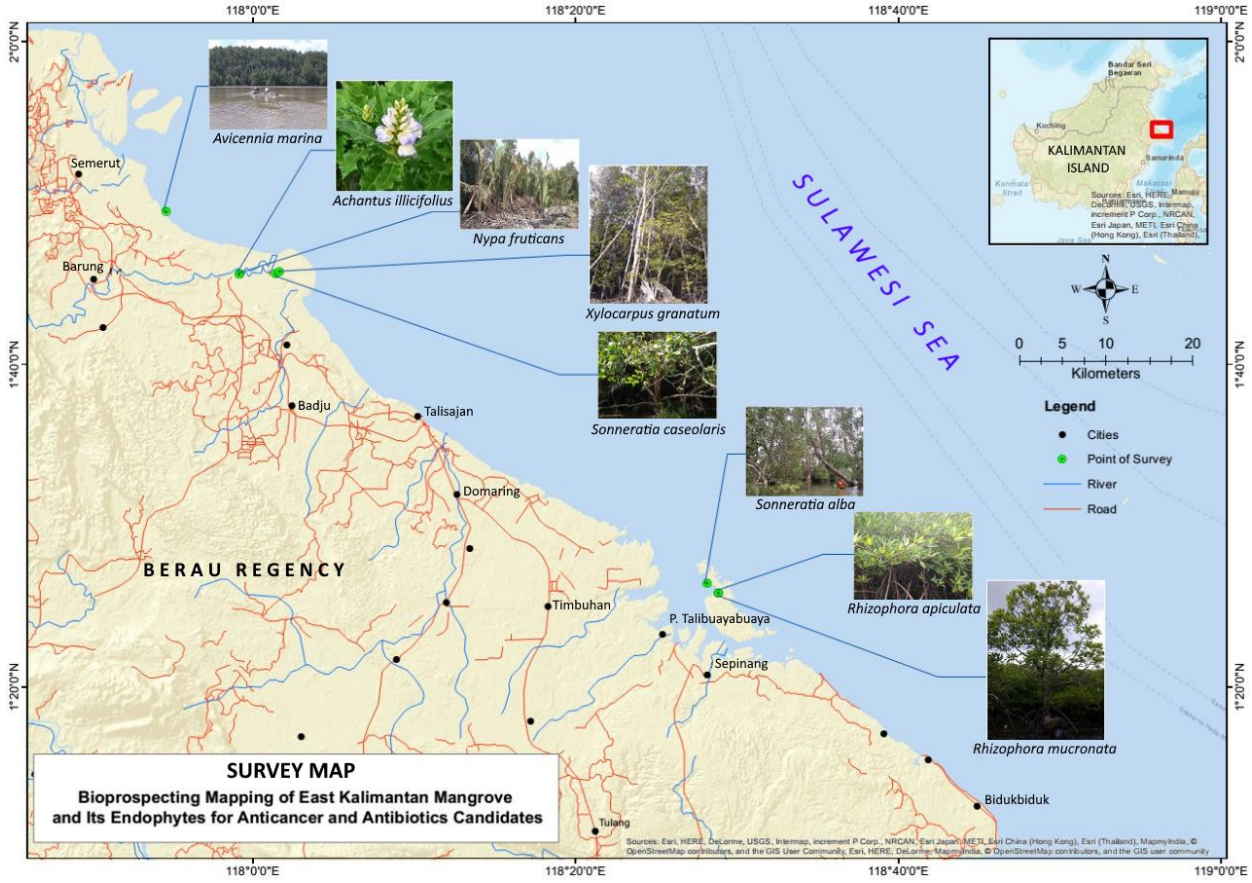

Figure 1. Map of sampling area

After 14-day incubation, the extraction process was carried out by separating mycelia from the medium filtrate using filter paper vacuum filtration. The mycelia were then oven-dried at 60 ºC for 24 hours. The filtrate was extracted further using a separating funnel with 1:1 (v/v) of ethyl acetate (EtOAc) solvent and the culture medium; the procedure was repeated 3 times. The filtrate was evaporated using a rotary evaporator at 40–45 ºC until the liquid thickened. This crude fungal extract gave the final yield which was subsequently weighed and tested for its antibacterial activity using the agar disc diffusion method. The MIC and MBC values were determined using the microdilution method.

Antibacterial activity test

The test aimed to determine the growth inhibitory activity of the crude extract of the endophytic fungi isolates against the pathogenic bacteria: *E. coli* ATCC 25922, *S. aureus* ATCC 25922, *B. cereus* ATCC 10876, and *S. typhimurium* ATCC 14028. The test bacterial suspension (1.5×10^8 cfu mL^{-1}) were inoculated by rubbing evenly onto the surface of Mueller Hinton Agar (MHA) medium using sterile swabs. Crude extracts were prepared at the concentration of 10 mg mL^{-1} using DMSO as solvent. Sterile disc paper with a diameter of 6 mm was given a drop of 20 µL of the crude fungal extract. Then the disc paper was placed on the MHA media inoculated with bacterial suspension (1.5×10^8 cfu mL^{-1}). Following 37 ºC incubation for 24 hours, inhibition zone, including the crude extracts disc's diameter, was compared to those of the control (chloramphenicol). Antibacterial activity was determined by observing the formation of an inhibition zone or clear zone (mm) around the paper disk. Paper disks treated with DMSO (20 µL) and chloramphenicol (50 µg mL^{-1}) served as negative and positive controls, respectively. The test was carried out in triplicate for each extract.

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values aimed to determine the minimum concentration of crude antibacterial extract in inhibiting and killing the tested bacteria, respectively. The microdilution method was carried out using a 96-well microtiter plate, in which the first column wells were filled with 100 µL of Mueller Hinton Broth (MHB) medium and 100 µL of the crude fungal extract (10 mg mL^{-1}). Then, 100 µL mixture was extracted from the first column wells and transferred to the second column of wells already containing 100 µL of MHB. This gradual dilution operation was repeated column by column till the second last column, resulting in an array of columns containing the antibacterial extract with a decreasing concentration of 10, 5.0, 2.5, 1.25, 0.625, 0.312, and 0.156 mg mL^{-1}. Then, 100 µL of test bacteria suspension (1×10^6 CFU mL^{-1}) was pipetted into each column well, incubated at 37 ºC for 24 hours. The MIC value was determined based on the absence of test bacteria growth indicated by no turbidity at the lowest antibacterial extract concentration. The samples contained in these no-turbidity wells were used to determine the value of MBC by plating 0.1 of them on MHA medium plates, incubated at 37 ºC for 24 hours. The MBC value was determined based on the petri dish not overgrown with the colonies.

Toxicity test

The toxicity test was determined using Brine Shrimp Lethal Test (BSLT). A total of 10 healthy *Artemia salina* larvae (based on their motility and light-seeking ability) were put into a bottle vial containing 100 µL of artificial seawater. The fungal extract solution was added at different concentrations (1000, 100, and 10 ppm) to each bottle vial with control vial having no extract solution added. The test and done in replicate for each treatment. After 24-hour observation, the numbers of dead *Artemia* larvae were counted using a magnifying glass. The LC_{50} value was then obtained based on the calculation of the percentage value of shrimp larvae mortality:

$$\%\ Mortality = \frac{Total\ dead\ larvae}{Total\ larvae\ tested} \times 100\%$$

LC-MS analysis of secondary metabolites

The crude fungal extract (10 mg) was dissolved in 5 mL methanol and filtered through a 0.2 µm PTFE membrane. The secondary metabolite analysis of the extract

was carried out using LC-MS or UHPLC Tandem Q-Exactive Plus Orbitrap HRMS (Thermo Fischer Scientific) with an Accurcore column C18, 100 × 2.1 mm, 1.5 µm. Water rate was set at 0.2 mL/minutes. Two eluents were prepared: H_2O + 0.1% formic acid (eluent A) and Acetonitrile + 0.1% formic acid (eluent B). Gradient elution was programmed as follows: 0–1 minute (5% B), 1–25 minutes (5–95% B), 25–28 minutes

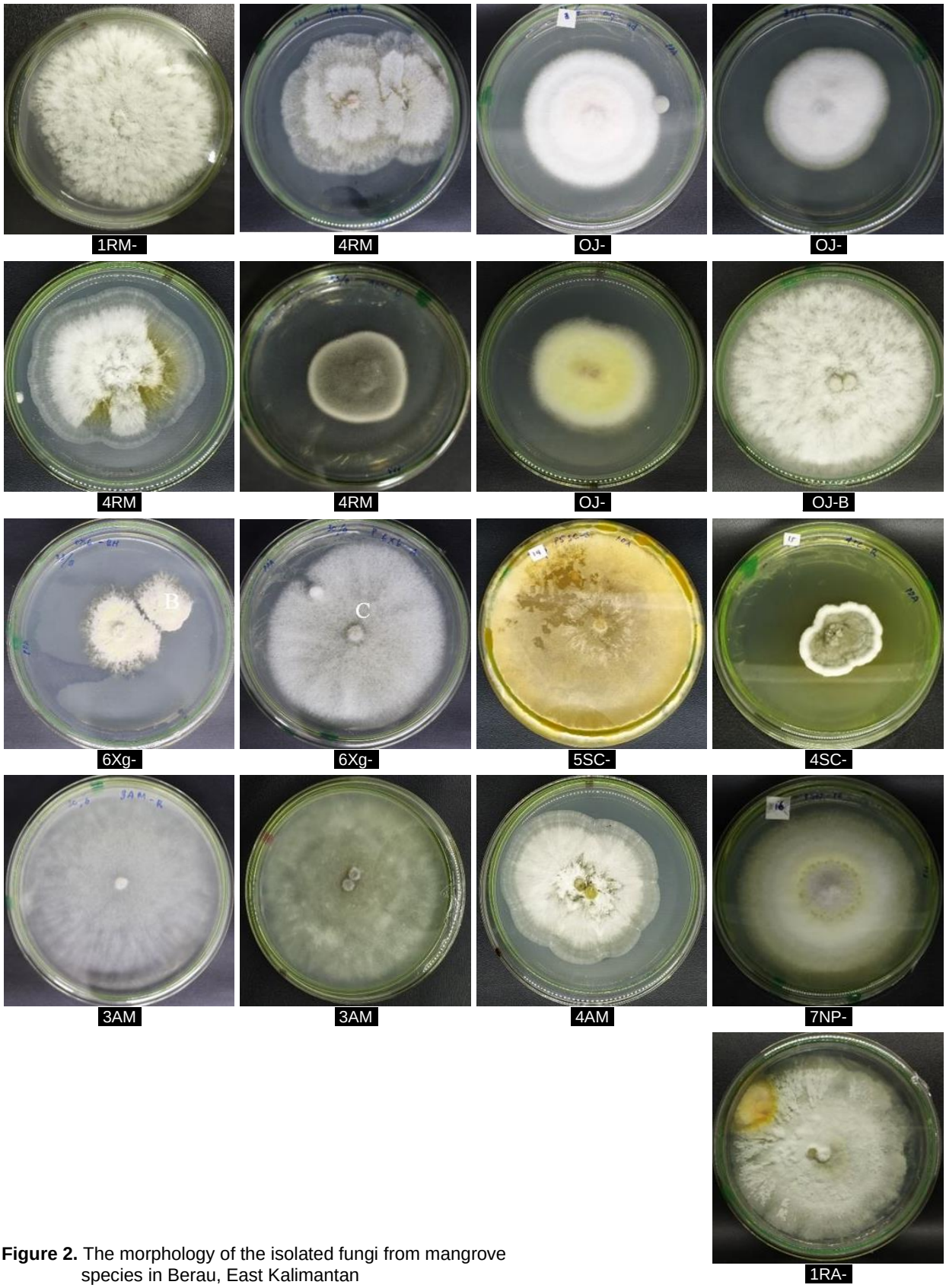

Figure 2. The morphology of the isolated fungi from mangrove species in Berau, East Kalimantan

(95% B), and 28–30 minutes (5% B) with 30 ºC column temperature, 2 µL injection volume, 100–1500 m/z mass range, and positive ionization mode.

Molecular identification of fungi

Selected fungi were molecularly identified using ITS (internal transcribed spacer) as a molecular marker. The mycelia were ground with liquid nitrogen to a fine powder to obtain pure DNA extract for amplification using the primers ITS 1 and ITS 4 (Nurhalimah et al. 2021). The 35-cycle amplification was carried out at four stages: pre-denaturation (95 ºC, 1 minute), denaturation (95 ºC, 15 seconds), annealing (52 ºC, 15 seconds), and extension (72 ºC, 45 seconds). The PCR product (7 µL) was electrophoresed in 1% agarose for 30 minutes and then stained using SYBRSafe (Invitrogen) for 25 minutes, followed by observation under a UV transilluminator lamp.

The purified PCR product was sent to First Base Laboratories Sdn Bhd (1st BASE), Malaysia. The subsequent kinship analysis was carried out using the BLAST (Basic Local Alignment Search Tool) database application at NCBI (National Center for Biotechnology Information). A phylogenic tree was constructed using the MEGA X Neighbor-Joining 1000 bootstrap.

The research method used was experimental laboratories using the basic research design of Completely Randomized Design (CRD) and further testing with BNT (Least Significant Difference)

RESULTS AND DISCUSSION

Endophytic fungal isolates

A total of 17 strains (Figure 2) were obtained from the root, stems, leaves, fruit, and flowers of 7 mangrove species. Each isolate showed different morphological characteristics such as texture, growth pattern, surface color, topography, and exudate droplets (Table 1).

Li et al. (2016) isolated endophytic fungi from 4 mangrove species, namely *Aegiceras corniculatum* (12 isolates from leaves and 17 isolates from twigs), *A. marina* (2 isolates from leaves and 16 isolates from twigs), (*Bruguera gymnorrhiza*) (6 isolates from leaves and 16 isolates from twigs), and *Kandelia candel* (21 isolates from leaves and 42 isolates from twigs). The diversity of isolated fungi could be affected by the seasonal factor. The summer season has a higher fungi species diversity index than winter does. This suggests that humidity and high temperatures during summer support the growth of endophytic fungi in colonizing plants. The environmental condition of the habitat of the host plants is a factor that influences the structure and composition of the endophytic microbial species associated with the roots, stems, twigs, and leaves. This condition indicates that the presence of various types of endophytic microbes depends on the interaction between endophytic microbes or the interaction of endophytic microbes with pathogens (de Souza Sebastianes et al. 2013). The type of mangrove host plant also affects the growth of fungi. The similarity of the endophytic species that grow is relatively low even though the mangrove plants grow in the same area (Costa et al. 2012).

Fermentation and extraction

The yield of the endophytic fungi extracts is shown in Figure 3. Based on the results, OJ-B and OJ-BG isolates showed the highest (19.20 ± 1.10 mg) and the lowest (10.50 ± 0.06 mg) extract yields, respectively, in 100 mL PDB media. In a previous study involving mangrove endophytic fungi, Rahmawati et al. (2019) obtained the extract yield as much as 14, 12, and 17 mg from the root, stems, and leaves, respectively. Meanwhile, the mangrove species *X. granatum* yielded 15 and 18 mg of endophytic fungi extract from the stems and 17 mg from the leaves.

Analysis of antibacterial extract

The antibacterial activity test was conducted using 20 µL of the crude extract and showed that all the endophytic fungi isolates demonstrated inhibitory activity against the pathogen bacteria (Table 2). Notably, 3 endophytic fungi (OJ-B, OJ-Bu, and 6Xg-Bh) showed relatively higher inhibition zones (Table 3).

Table 1. Morphological characters of endophytic fungi on the fruits, flowers, leaves, stems and root of the several mangroves

No	Isolate Code	Morphological Characters
1	1RM-B4	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
2	4RM-B	Circular growth pattern, mycelia like cotton, mycelia like cotton, the opposite color yellowish in the center and white at the edges, forming a radial line, forming concentric circles
3	4RM-R	Circular growth pattern, mycelia like cotton, mycelia like cotton, the opposite color yellowish in the center and white at the edges, forming a radial line, forming concentric circles
4	4RM-D	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
5	3AM-B	Circular growth pattern, mycelia like velvet, surface color grayish white, grayish white by forming a black ring, forming radial lines and concentric circles
6	3AM-R	Circular growth pattern, mycelia like velvet, surface color grayish white, grayish white by forming a black ring, forming radial lines and concentric circles
7	4AM-B	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
8	OJ-Ad	Circular growth pattern, mycelia like cotton white, white color surface, yellowish white reverse color, forming radial lines and concentric circles
9	OJ-BG	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
10	OJ-BU	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
11	OJ-B	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
12	6Xg-Bh	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles
13	6Xg-A	Circular growth pattern, mycelia like velvet, surface color grayish white, grayish white by forming a black ring, forming radial lines and concentric circles
14	5SC-B	Circular growth pattern, mycelia like cotton white, yellow surface color yellow-orange reverse color, not form radial lines and concentric circles
15	4SC-R	Circular growth pattern, mycelia like cotton white, surface color gray with white edges, color gray by forming a black ring, not forming radial lines and concentric circles
16	7NP-B	Circular growth pattern, mycelia like cotton white, white color surface, center point yellowish, yellowish reverse color, forming radial lines and concentric circles
17	1RA-BK	Circular growth pattern, mycelia like cotton, mycelia like cotton, yellowish white reverse color, forming radial lines and concentric circles

The highest antibacterial activity was shown by OJ-B crude extract with the highest inhibition zone on gram-positive bacteria (*S. aureus* and *B. cereus).* Our finding was coherent with the results obtained by Anindyawati and Priadi (2017), who stated that the endophytic fungi extract from star fruit plants had a much larger zone of inhibition against gram-positive bacteria than the zone produced by gram-negative bacteria. This is probably due to the different cell structures between gram-positive and gram-negative bacteria.

Handayani et al. (2019) tested the antibacterial activity of the crude extracts of mangrove endophytic fungi of which *Sonneratia alba* showed the highest inhibitory activity against *S. aureus* bacteria and *E. coli* at 11.03 and and 12.38 mm inhibition zones, respectively. Similar study by Santoso et al. (2015) also reported the metabolite extract of endophytic fungi from mangrove *R. apiculata* being inhibitory against the growth of *S. aureus* (22.6 mm inhibition zone) and *P. aeruginosa* (23.3 mm inhibition zone). The condition of the

Table 2. Inhibition zone (mm) of the metabolite crude extract (10 mg mL^{-1}) by the disc-diffusion method

Crude Extract	Inhibition zone (mm)			
	S. aureus	*E. coli*	*B. cereus*	*S. Typhimurium*
1RM-B4	7.10 ± 0.70	–	8.13 ± 2.49	8.60 ± 3.90
4RM-B	–	–	–	–
4RM-R	–	–	–	–
4RM-D	–	–	–	–
3AM-B	–	–	–	–
3AM-R	–	8.6 ± 1.99	–	10.13 ± 3.28
4AM-B	8.17 ± 1.42	–	8.77 ± 3.84	10.67 ± 4.38
OJ-Ad	12.50 ± 5.45	–	8.73 ± 3.69	9.40 ± 4.94
OJ-BG	8.45 ± 0.90	–	8.33 ± 2.85	7,00 ± 0.70
OJ-BU	22.13 ± 1.58	16.60 ± 1.27	13.73 ± 2.03	10.67 ± 0.80
OJ-B	21.30 ± 0.98	19.47 ± 2.96	25.40 ± 2.28	15.93 ± 2.67
6Xg-Bh	20.20 ± 3.42	9.63 ± 0.75	11.60 ± 3.20	12.30 ± 2.78
6Xg-A	–	7.20 ± 1.12	–	7.56 ± 3.28
5SC-B	–	7.23 ± 1.44	–	7.40 ± 1.60
4SC-R	–	7.10 ± 0.87	–	8.43 ± 3.09
7NP-B	–	–	–	–
1RA-BK	–	–	–	–
K (+)	34.63 ± 0.75	33.13 ± 0.05	35.43 ± 2.59	33.80 ± 0.50
K (-)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

RM: *Rhizopora mucronata*; AM: *Avicennia marina;* OJ: *Achantus illicifolius;* Xg: *Xylocarpus granatum*; SC: *Sonneratia caeseolaris*; NP: *Nypa frutican;* RA: *Rhizopora apiculate;* Bh: Fruit, B: Steams, R: Twig, D: Leaves, BG: Flowers, Bu: Fruit

Table 3. High inhibition zone of three crude extract highest

Crude Extract	Inhibition Zone (mm)			
	S. aureus	*E. coli*	*B. cereus*	*S. typhi*
6Xg-Bh	20.20 ± 3.42^{a}	9.63 ± 0.75^{a}	11.60 ± 3.20^{a}	12.30 ± 2.78^{a}
OJ-Bu	22.13 ± 1.58^{bc}	16.60 ± 1.27^{b}	13.73 ± 2.03^{ab}	10.67 ± 0.80^{b}
OJ-B	21.30 ± 0.98^{ab}	19.47 ± 2.96^{c}	25.40 ± 2.28^{c}	15.93 ± 2.67^{c}
Control (+)	34.63 ± 0.75^{d}	33.13 ± 0.05^{d}	35.43 ± 2.59^{d}	33.80 ± 0.50^{d}
Control (-)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Value was mean ± SD (standard Deviation) from three replication; Control (+): chloramphenicol; Control (-): DMSO; (a, b, c, and d): different letter in the same column represent significance

mangrove's diverse ecosystem causes mangrove fungi to live in an extreme environment. This situation potentially produces numerous compounds, including bioactive metabolites. Secondary metabolites produced by endophytic fungi respond to environmental stress, so different environments may affect the condition of endophytic fungi in producing secondary metabolite compounds. Other conditions such as salinity, pressure, and temperature in marine organisms can have different and unique natural product potential in each species (Hamed et al. 2015). In addition, factors such as season, age, environment, and location can affect the biological compounds produced by endophytic fungi (Strobel et al. 2005).

Based on the results, the MIC values in the three crude extracts of endophytic fungi were between 0.625 to 10 mg mL^{-1}. The highest minimum inhibition of the three extracts was shown by OJ-B isolate, which was 0.625 mg mL^{-1} in all test bacteria.

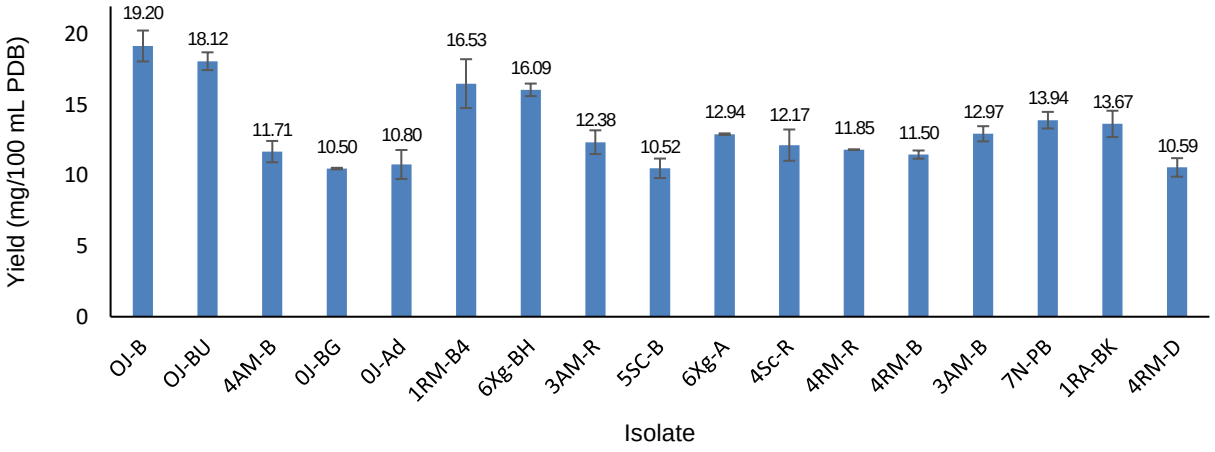

Figure 3. Extract yield of secondary metabolites endophytic fungi mangrove

Table 4. MIC, MBC and toxicity of the metabolite crude extract (10 mg mL^{-1})

Crude extract	MIC (mg mL^{-1})				MIC (mg mL^{-1})				Toxicity	
	SA	EC	BC	ST	SA	EC	BC	ST	LC_{50} (ppm)	Criteria
6Xg-Bh	2.5	2.5	2.5	5	5	10	10	10	1291.291	not toxic
OJ-Bu	1.25	1.25	1.25	1.25	5	5	10	10	1807.174	not toxic
OJ-B	0.625	0.625	0.625	0.625	2.5	2.5	5	5	1042.317	not toxic
Control (+)	0.15	2.50	0.31	10	0.31	2.50	0.31	0.31	–	–
Control (-)	–	–	–	–	–	–	–	–	–	–

(-): no activity; Control (+): chloramphenicol; Control (-): DMSO; SA: *S. aureus;* EC: *E. coli;* BC: *B. cereus;* ST: *S. typhi*

Meanwhile, the MBC value of the endophytic fungi extract was between 2.5 to 10 mg mL^{-1}, with the highest bacterial mortality rate indicated by the OJ-B fungi extract at 2.5 mg mL^{-1} and the OJ-Bu extract at 10 mg mL^{-1} in the test bacteria *S. aureus* and *E. coli*. These results follow the research of Sangkanu et al. (2017), which states that the bactericidal value of an antimicrobial compound will have 2-4 times greater than the minimum inhibition value. The research of Cai et al. (2017a) also showed that MIC values of the endophytic fungi *A. ilicifolius* extract of 5.60 and 11.21 g mL^{-1} on the inhibition of *B. subtilis* and *P. aeruginosa* test bacteria. While the research of Chen et al. (2016a) with the same extract from the endophytic fungi *A. ilicifolius* produced MIC values between 1.6 and 13 g mL^{-1} in *S. aureus* test bacteria. Cai et al (2017b) showed that extracts from mangrove endophytic microbes, especially fungus *A. licifolius* had MIC values of 8.0 and 0.25 g mL^{-1}, which was indicated by the biofilm penetration activity, which inhibited the growth of *S. aureus* and *B. subtilis.*

According to the result of OJ-B isolates, namely endophytic fungi, isolated from mangrove stem of *A. ilicifolius* species had the highest antibacterial activity compared to other isolates. This finding in accordance with the statement of Firdaus et al. (2013), which stated that the mangrove species *A. ilicifolius* is a plant that is efficacious in treating local communities, especially for the treatment of antibacterial, asthma, and even rheumatism. The antimicrobial activity of this mangrove extract showed inhibition against *B. subtilis, S. aureus, P. aeruginosa,* and *Proteus vulgaris* bacteria. Research from Chen et al. (2016b) also showed significant antibacterial activity of *A. ilicifolius* mangrove stems against *B. subtilis, P. aeruginosa,* and *S. aureus* bacteria.

Analysis of toxicity of three crude extracts shown that three of all extract not toxic. The regression results of the correlation coefficient value (R2) showed the value almost close to 1, meaning that the data obtained was good. The LC_{50} values (Table 4) in the three samples were 1042.317

Table 5. Chemical compound crude extract OJ-Bu with LC-MS

RT (min.)	Mol. weight (m/z)	Compound	Formula	ChemSpider Result
0.990	103.0997	Choline	$C_5H_{13}NO$	19
1.043	143.0946	DL-Stachydrine	$C_7H_{13}NO_2$	54
1.395	123.0320	Nicotinic acid	$C_6H_5NO_2$	9
1.455	169.0738	Pyridoxine	$C_8H_{11}NO_3$	20
2.021	167.0946	Anhydroecgonine	$C_9H_{13}NO_2$	54
2.770	231.0466	Pyrazol-5(4H)-one, 1-(2-benzothiazolyl)-3-methyl-	$C_{11}H_9N_3OS$	9
5.388	196.1211	3-(propan-2-yl)-octahydropyrrolo[1,2-a] pyrazine-1,4-dione	$C_{10}H_{16}N_2O_2$	26
5.520	129.0578	Quinoline	C_9H_7N	7
8.308	244.1211	Cyclo(phenylalanyl-prolyl)	$C_{14}H_{16}N_2O_2$	45
10.466	226.147	N-Isopropyl-N'-phenyl-p-phenylenediamine	$C_{15}H_{18}N_2$	13
12.165	299.0793	1-[2-(2-Naphthyloxy) acetoxy]-2,5-pyrrolidinedione	$C_{16}H_{13}NO_5$	19
12.749	236.1160	hexobarbital	$C_{12}H_{16}N_2O_3$	32
14.978	279.2550	Linoleamide	$C_{18}H_{33}NO$	3
15.112	431.1944	Fusarin C	$C_{23}H_{29}NO_7$	2
16.256	281.2718	Oleamide	$C_{18}H_{35}NO$	7
19.430	370.1681	1-(o-Benzoylbenzoyl)-4-phenylpiperazine	$C_{24}H_{22}N_2O_2$	3
19.994	489.3090	Borrelidin	$C_{28}H_{43}NO_6$	2
20.545	290.1783	azatadine	$C_{20}H_{22}N_2$	8
20.744	294.2194	9-Oxo-10(E),12(E)-octadecadienoic acid	$C_{18}H_{30}O_3$	48
23.901	783.4094	Beauvericin	$C_{45}H_{57}N_3O_9$	2
24.433	386.2722	5-Hexyl-2-(4'-pentyl-4-biphenylyl) pyrimidine	$C_{27}H_{34}N_2$	3

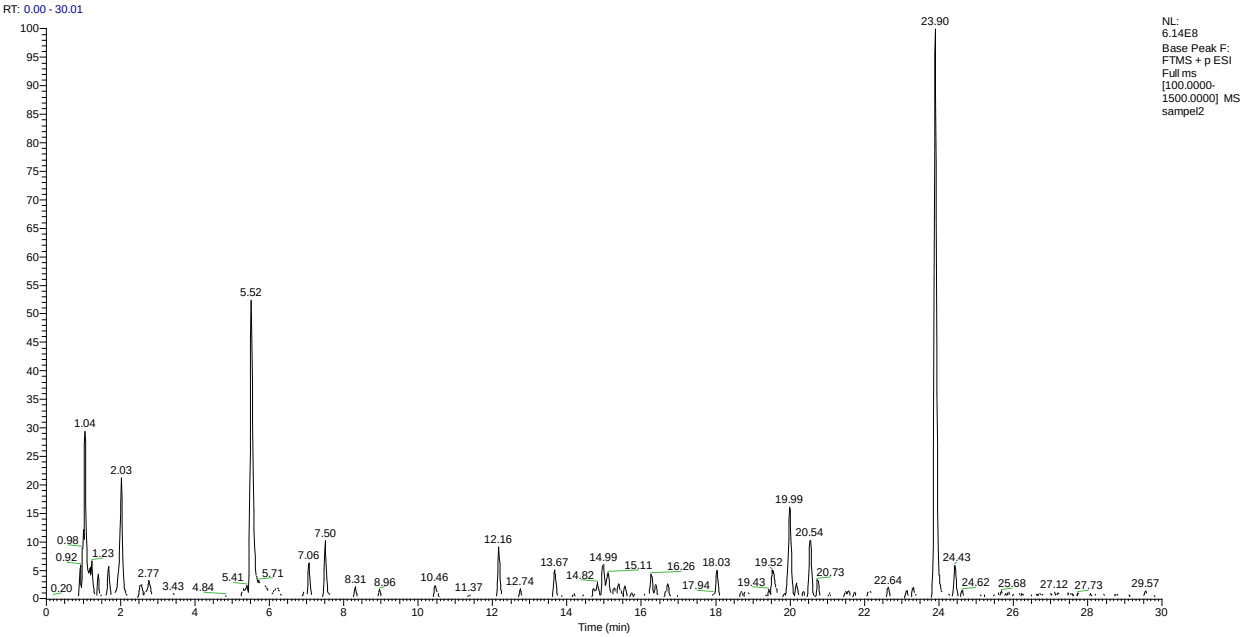

Figure 4. Chromatogram LC-MS of crude extract metabolites OJ-B

ppm OJ-B extract, 1807.174 ppm OJ-Bu extract, and 1291.291 ppm 6Xg-Bh extract. Toxicity is defined as how active a compound is in causing damage to the test organism. According to Setyowati (2016), LC_{50} values > 1000 ppm are categorized as non-toxic, and if < 100 ppm are categorized as very toxic. Based on the calculation results, the three

samples were categorized as non-toxic. The lower the ppm value of the compound extract produced, the greater the inhibition zone value when antimicrobial testing is carried out. This is in accordance with Setyowati's (2016) research, where the BSLT test of the extract of the seagrass compound *Enhalus acoroides* showed that the larger the inhibition zone produced by the antibacterial, the more toxic the compound with a lower ppm value. Based on the BSLT test data from the three samples, which had a high inhibition zone and the toxicity value indicated non-toxic, it was decided that only one sample would proceed to the crude extract profiling test of secondary metabolites, which was OJ-B crude extract.

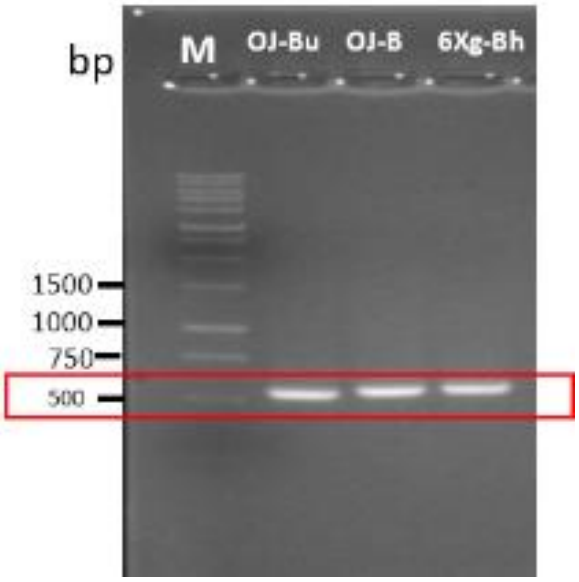

Figure 5. PCR product from endophytic fungi OJ-B, OJ-Bu and 6Xg-Bh with ITS 1 and ITS 4; DNA mareker 1 Kb

Identification of chemical compounds

Result of analysis of chemical compound profile from crude extract of endophytic fungi OJ-B (Table 5) and results of chromatogram LC-MS (Figure 4). Several chemical compounds are supposed of having antibacterial. These compounds are betaine, which can inhibit the growth of *Pseudomonas aeruginosa, S. epidermidis, E. coli, S. aureus,* and *Candida albicans* (Liu et al. 2013). Proline has antibacterial activity and is safe if applied to food products with a certain amount (Scocchi et al. 2011). Adenosine can inhibit the growth of *C. albicans* and *E. coli* (Bakr et al. 2021). L-Phenylalanine inhibits the growth of Gram-positive bacteria (Peng et al. 2021). Quinoline (O'Donnell et al. 2010) inhibited Gram-positive bacteria, especially *S. aureus*. Cyclo(phenylalanyl-prolyl) has antibacterial, antifungal, antivirus. Cyclo compounds (phenylalanyl-prolyl) are usually produced by bacteria lactic acid (Ivanov et al. 2021). Borrelidin has excellent antifungal activity successfully isolated from soybean roots (Zhang et al. 2018).

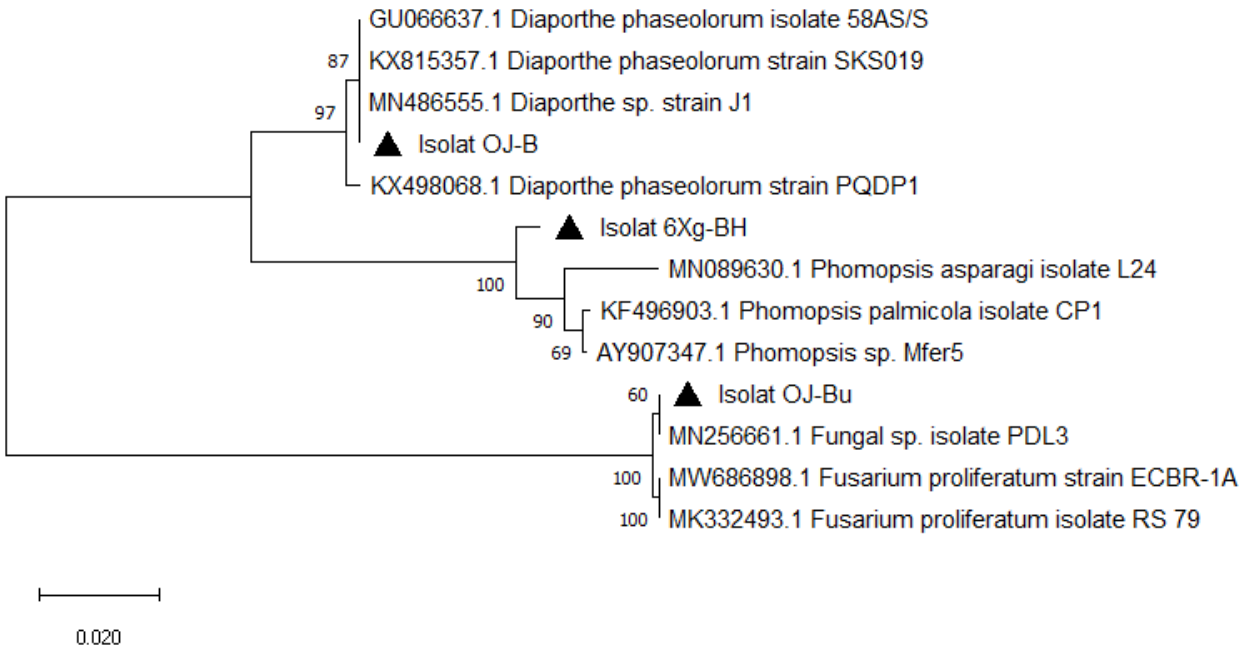

Figure 6. Phylogenetic tree of OJ-B, OJ-Bu and 6Xg-Bh constructed with the aid of MEGA X program

Compounds that appear in the highest chromatogram peak (Figure 4) are Beauvericin. The possibility of active compounds that play a role in the activity of antimicrobials from the extraction of OJ-B endophytic fungi isolates extracted in the medium is Beauvericin. According to Wang et al. (2012), the compound beauvericin has antimicrobial, insecticidal, and antitumor activity. The antimicrobial activity of beauvericin can inhibit the Gram-positive bacteria *Bacillus spp*., *Bifidobacterium adolescentis, Clostridium perifringens, Enterococcus faecium, Eubacterium biforme, Listeria monocytogenes, Mycobacterium tuberculosis, Peptostreptococcus spp., Staphylococcus haemolyticus, Paenibacillus spp*. Meanwhile, Gram-negative bacteria that can be inhibited include: *Agrobacterium tumefaciens, E. coli* CECT 4782, *E. coli*, *P. aeruginosa, P. lachrymans, S. enterica, Shigella dysenteriae, Xanthomonas vesicatoria, Yersinia enterocolitica.* Beauvericin is a bioactive compound found in fungi, especially the types of fungi *Beaveria bassiana* and *Fusarium spp.*

Molecular identification

Molecular identification of the three selected isolate samples was carried out using PCR and ITS markers. The results of the PCR product were followed by electrophoresis to prove whether the PCR product was perfectly amplified with an indicator of the formation of a single band that was parallel to the DNA marker. Based on the results of single bands on agarose gel by electrophoresis, the three isolates were amplified at a length of ± 500-600 bp (Figure 5).

The partial sequence of the ITS rRNA gene for endophytic fungi was compared with the gene sequence database in *Gen bank* BLAST. The results showed that OJ-Bu isolate was related to *Fusarium proliferatum* similarity 99.82%, OJ-B was related to *Diaporthe phaseolorum* similarity 99.83%, and the 6Xg-Bh isolate was associated with *Phomopsis palmicola* similarity 98.15%. Three isolates had a homology percentage exceeding 98%. According to Setyati et al. (2019), the similarity value of the genetic sequence if ≥ 97% indicates identical species. The phylogenetic tree construction is shown in Figure 6.

The results of the phylogenetic tree construction using MEGA X software with the Neighbor-Joining method showed that OJ-B isolates were in the same cluster with *D. phaseolorum* species, OJ-Bu isolates were in one cluster with *F. proliferatum* species, and 6Xg-BH isolates were in the same cluster with *Phomopsis palmicola* isolates. If they are in one cluster, it can be concluded that the isolates are closely related. This result is also evidenced by the percentage value of homolog identity, more than 98%.

CONCLUSION

Seventeen endophytic fungi were successfully isolated from seven species of mangroves from Berau, East Kalimantan, Indonesia. Three extracts from isolates OJ-B, OJ-Bu, and 6Xg-Bh showed minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) ranging from 0.625 – 5 mg mL^{-1} and 2.5 – 10 mg mL^{-1}. The chemical identification using liquid chromatography-mass spectrometry (LC-MS) showed potential antibacterial compounds from isolate OJ-B namely beauvericin, choline, nicotinic acid, pyridoxine, quinoline, cyclo (phenylalanyl-prolyl), fusarin c, oleamide, and borrelidin. At the same time, the molecular based identification of the fungus revealed three species, namely *D. phaseolorum*, *F. proliferatum,* and *Phomopsis* sp. This research discovers sustainable use of species, especially endophytic fungi from mangroves, as material from bioproduction of antibacterial.

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