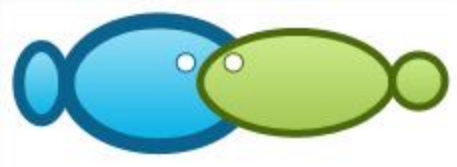

Molecular identification and antibacterial activity of marine-endophytic fungi isolated from sea fan *Annella* sp. from Bunaken waters, Manado, North Sulawesi, Indonesia

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Abstract. This study aims to analyze the antibacterial activity and identify endophytic fungi in sea fan *Annella* sp. from Bunaken waters, Manado, Indonesia. Research procedures started from isolation, purification, and cultivation of the fungi. Extraction utilized ethyl acetate (EtOAc) for media broth and methanol (MeOH) for fungal mycelia. Antibacterial testing used the agar diffusion method. EtOAc extract and MeOH extract were then tested to pathogen *Staphylococcus aureus,* Methicillin Resistant *Staphylococcus aureus* (MRSA), and *Escherichia coli*. Molecular identification was carried out from the DNA extraction, PCR process, electrophoresis, and sequencing. The primers used were DNA Internal Transcribed Spacer (ITS) region, ITS1 (5′-TCC GTA GGT GAA CCT GCG-3 ′) and ITS4 (5′-TCC TCC GCT TAT TGA TGA TAT GC-3 ′). The results showed that endophytic fungi were identified as *Xylaria feejeensis, Daldinia eschscholtzii,* and *Penicillium citrinum*. The three endophytic fungi isolates have high antibacterial inhibition potential against Gram-positive *S. aureus,* MRSA and Gram-negative *E. coli* so that they can be a source of new antibacterial compounds.
Key Words: agar diffusion, DNA ITS región, inhibition potential, gram-positive, gram-negative, bacteria.

Introduction. Infectious diseases are one of the causes of death around the world from pathogenic microbes. Antibiotics are used to treat infectious diseases, but their improper application can make microbes be resistant to them. Resistant bacteria include Methicillin-Resistant *Staphylococcus aureus* (MRSA), Multidrug-Resistant Tuberculosis (MDR-TB), Vancomycin-Resistant *Staphylococcus aureus* (VRSA), Multidrug-Resistant *Escherichia coli* (MDR-EC), Multidrug-Resistant *Staphylococcus aureus* (MDR-SA) (CDC 2019). Discovery of novel antibiotic compounds for broad-spectrum antimicrobial activity is very important because of the difficulty in curing infectious diseases caused by resistant bacteria. Marine-endophytic fungi are a rich source of active metabolite compounds (Zhang et al 2009).

Sea fans of genus Gorgonia are a member of the Anthozoa class, a sub-class of Octocorallia (Alcyonaria) that live attached to hard substrates such as coral reefs (Fabricus & Alderslade 2001). Gorgonians are known to have secondary metabolite compounds that have the ability to inhibit microbial growth. Research conducted by Rodriguez et al (2000), Fuganti & Serra (2000), Rodriguez et al (2003), Putri et al (2019), and Kandou et al (2019) found several types of gorgonians that have antibacterial activity. It is possible the activity of these antibacterial compounds is produced by endophytic microbes. According to Tan & Zou (2001), endophytic microbes produce bioactive compounds that have similar character as their host. Based on a number of studies, gorgonians have endophytic microbes in their tissues and produce

bioactive compounds that have antibacterial (Wang et al 2011), antiviral (Nong et al 2014), and antifungal (Dong et al 2014) activities. Gorgonian *Dichotella gemmacea* has the endophytic fungi *Aspergillus versicolor* that have the antibacterial activity against *Staphylococcus epidermidis* (Chen et al 2014a), *Cochliobolus lunatus* against *Staphylococcus aureus* (Shao et al 2011), and *Penicillium fungi* against *Bacillus subtilis, Escherichia coli, Micrococcus luteus,* and *Pseudoalteromonas nigrifaciens* (Bao et al 2013). Chen et al (2014b) isolated the fungal *Eurotium* from gorgonian *Subergorgia suberosa* from the South China Sea and had antibacterial activity against *Staphylococcus epidermidis* and *Bacillus cereus*. Wang et al (2011) isolated the fungal *Penicillium* from gorgonian *Echinogorgia rebekka* from the South China Sea which has high antibacterial activity against *Staphylococcus aureus, Micrococcus tetragenus*, and *Bacillus subtilis*. Gorgonian *Muricella abnormaliz* has the endophytic fungi *Penicillium commune* which has antibacterial activity against *Escherichia coli* and *Enterobacter aerogenes* (Wang et al 2012) and *Aspergillus* against *Staphylococcus epidermidis* and *Bacillus cereus* (Chen et al 2014c).

Bunaken waters, North Sulawesi, Indonesia, is one of the waters that has a high diversity of marine biota (MEFI 2018), thus providing opportunities for the discovery of various antimicrobial metabolites. Based on the preliminary survey conducted, it is known that Bunaken waters have various types of gorgonians (Kandou et al 2019; Putri et al 2019) but they have not yet been explored. Limited information about the types of endophytic fungi that live in gorgonians in the waters of Bunaken, North Sulawesi and their antibacterial activity indicated the necessity to identify and to assay their bioactivity. Encouraged by the idea of drugs from the sea, it is important to search and discover potential antibacterial compounds from marine biota. A number of studies have found bioactive compounds with new structures derived from the richness of marine life which has potential as medicinal preparations. The objectives of the study were to identify the types of endophytic fungi using the DNA Internal Transcribed Spacer (ITS) region and to analyze their antibacterial activity against Gram positive *Staphylococcus aureus,* Methicillin Resistant *Staphylococcus aureus* (MRSA), and Gram negative *Escherichia coli*.

Material and Method. This study was carried out from August to December 2019. Gorgonian *Annella* sp. samples (Figure 1) were collected from the waters of Alung Banua and Lekuan II in Bunaken waters, Manado, Province of North Sulawesi, Indonesia (Figure 2) on August 23-24, 2019. They were immediately put into sterile plastic ziplock bag and put in a coolbox at 4^{o}C, then taken to the Microbiology Pharmacy Laboratory, at the Sam Ratulangi University Manado for fungal isolation.

Figure 1. Gorgonian *Annella* sp. (original).

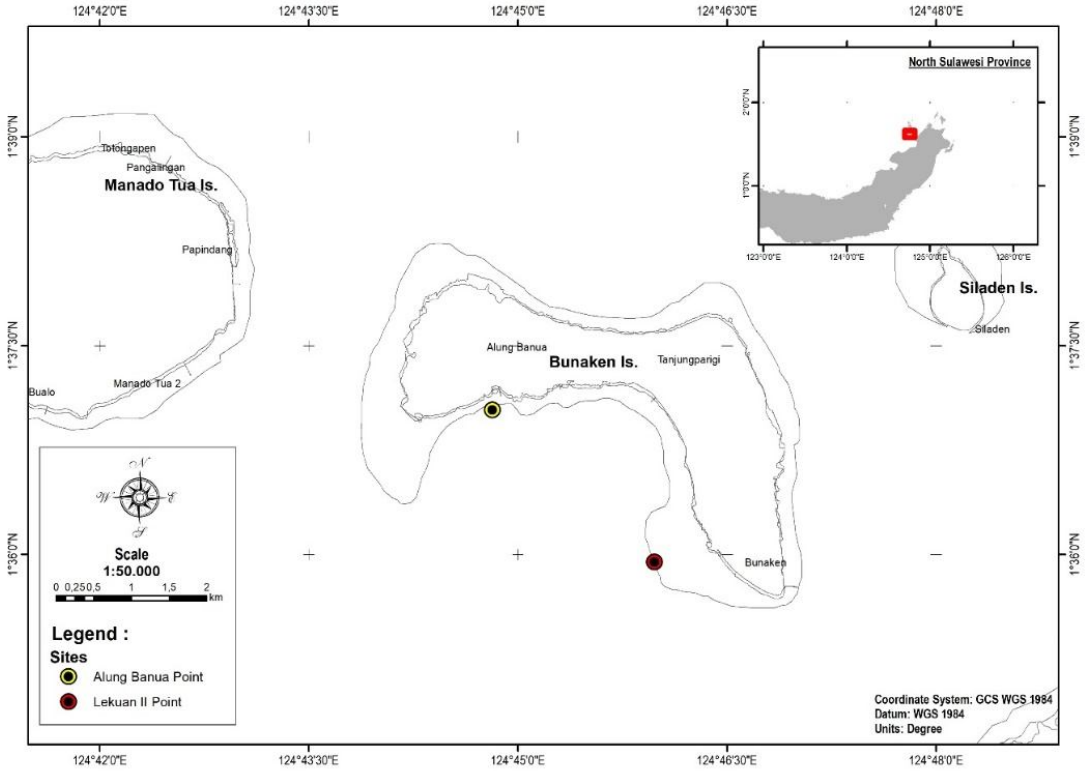

Figure 2. Map of sampling site.

Isolation and purification of endophytic fungi. Gorgonian samples were washed three times with sterile seawater to remove non-specific fungal propagules attached to the surface of gorgonian, then the samples were cut into 1 x 1 cm pieces and disinfected using 70% ethanol for 120 seconds for surface sterilization. Samples were inserted into a Potato Dextrose Agar (Merck®) plates (PDA+Chloramphenicol+sterile sea water 50%), incubated for 7 days at room temperature. Endophytic fungi growing on PDA were purified by inoculating a piece of hypha from each growth of different fungal colonies into new plates. Fungal cultures were incubated for 14 days at room temperature. Purification was based on macroscopic differences.

Morphological characterization of endophytic fungi. Characterization of endophytic fungi was carried out by observing the morphology of fungal colonies formed, the color of the colony, the reverse side color, the colony surface, and the diameter of the fungal colony growth.

DNA extraction using innuPrep DNA Micro Kit (Analityk Jena, Germany). The surface of the fungal colony was rubbed along 1 cm using a sterile inoculation needle then the tip of the needle was immersed in a 1.5 mL micro tube containing 200 µL buffer Lysis Solution TLS. The tube was inverted five times, incubated for 1 hour at 55°C using a TB2 thermoblock (Biometra) and then centrifuged at 11,000 rpm for 1 min. The supernatant was pipetted into a new microtube and mixed with 200 µL TBS Binding Solution then vortexed. The sample was put into a spin filter that has been facilitated with container and re-centrifuged. The filtrate was then discarded and the container was reassembled. A total of 400 µL Washing Solution HS was added and centrifuged at 11,000 rpm for 1 min. The filtrate was discarded and the container was reassembled. A total of 750 µL Washing Solution MS was entered and centrifuged. The filtrate was discarded and the container was reassembled. To dry the filter, the empty tube was recentrifuged for 2 min, and then the filtrate along with the container was discarded. The spin filter was moved into a new micro tube, dried for 2 min, added with 100 µL Elution Buffer, allowed to stand for 2 min and then centrifuged. The spin filter was removed and DNA was stored at -10ºC.

Polymerase chain reaction (PCR) for the amplification of fungal ITS DNA. PCR reaction used MyTaq™ HS Red Mix (Bioline). Each 40 µL reaction has 15 pmol from each primer and DNA template. The composition of the reaction was: 20 µL MyTaq HS Red Mix, 1.5 µL of each primer (10 µM), 15 µL ddH_2O, and 2 µL of template DNA. ITS DNA was amplified using PCR based on White et al (1990) with ITS1 primers (5′-TCC GTA GGT GAA CCT GCG-3′) and ITS4 (5′-TCC TCC TCC TCT TGA TGA TAT GC-3′). The reaction conditions for the first PCR used both primers: denaturation of 94°C (5 minutes) was followed with 35 cycles of denaturation of 95°C (30 seconds), annealing 52°C (30 seconds), extension 72°C (30 seconds).

Agarose gel electrophoresis. PCR results were separated using 0.8% agarose gel electrophoresis (in a 1x TBE buffer) and observed using UV-Transiluminator. As much as 0.8% agarose gel was made by boiling 0.8 g of agarose dissolved in 100 mL of TBE 1x. The hardened gel was immersed in 1x TBE solution. Agarose gel was printed and 10 µL of PCR products were loaded into gel wells. The gel has 100 Volt electricity for 30 min. The gel was soaked in a solution of ethidium bromide for 10 min. PCR product DNA was visualized using UV-Transiluminator and PCR success was detected in the presence of a single DNA band around 600 bp.

Sequencing. Sequencing uses two primers on the PCR. PCR results and both primers were sent to First Base CO (Malaysia) for sequencing. The results are in the form of chromatograms containing DNA sequences. The DNA sequences obtained were edited and compared using the ClustaW algorithm. Identification used the GenBank database (www.ncbi.nlm.nih.gov). The phylogenetic tree was created using Geneious v5.6 software with the Neighbor-Joining algorithm.

Cultivation and extraction of endophytic fungi. Pure fungi were propagated by inoculating 1.5 x 1.5 cm pieces of mycelia into an Erlenmeyer flask containing 100 mL of Potato Dextrose Broth (Merck®) + 50% sterile seawater, then shaken at 180 rpm on shakers at room temperature for 14 days. After 14 days of fermentation, the culture was centrifuged at 5000 rpm for 10 min at 40^{o}C to separate the fungal mycelia from the broth medium. Mycelia was extracted in 100 mL of methanol (MeOH) for 24 hours and the broth was extracted in 150 mL of ethyl acetate (EtOAc). These were carried out three times to obtain methanol extract and ethylacetate extract. Both extracts were evaporated at 40°C to get a crude extract (Zhang et al 2009). Extraction of fungal mycelia was done to secrete intracellular metabolites to the methanol solvent, while extraction of broth medium was conducted to attract extracellular metabolites from the broth medium to the ethylacetate solvent.

Antibacterial activity assay. Antibacterial activity assay against Gram positive *S. aureus,* MRSA, and Gram negative *E. coli* used the agar diffusion method (Kirby-Bauer). Bacteria were suspended using Nutrient Broth (NB) and incubated for 24 hours at 37^{o}C, standardized using a McFarland 0.5 which is equivalent to a bacterial density of 10^8 CFU mL^{-1}. A total of 1 mL bacteria was entered into 100 mL of Muller Hinton Agar (MHA), then poured into a sterile Petri dish aseptically. For antibacterial assay, 1 mg of methanol mycelia extract and 1 mg of etylacetate broth extract were dissolved in Dimethyl sulfoxide (DMSO), respectively. Aseptically, 50 µL of the extract was dropped on a sterile paper disk (6 mm, Whatman) and placed on the surface of MHA medium. Furthermore, the plates were incubated for 24 hours and the zone of inhibition was measured (three replications). Chloramphenicol antibiotics were used as positive control and DMSO as negative controls.

Results and Discussion. The purification obtained four isolates of endophytic fungi, isolates FKF1, FK511P, FK511H and FK5112H. Characterization was carried out by observing the morphological fungal colonies. Macroscopic characteristics of isolate FKF1 showed white color of colony and the reverse side, thickening hyphae and cotton-like elongated growth with 5-6 cm colony after 7 days of cultivation on Potato Dextrose Agar

plates. The characteristics of isolate FK511P were gray colony color and black on the reverse side, the hairy hyphae, mountainous surface, colony diameter 7 cm after 7 days of cultivation on Potato Dextrose Agar plates. The characteristics of FK511H were dark green hyphae and dark yellow on the reverse side, the hairy hyphae, colony diameter 1-2 cm after 7 days of cultivation on Potato Dextrose Agar plates. The characteristics of isolate FK5112H were greenblack in center and white on margins and brown on the reverse side color, colony diameter 3-4 cm after 7 days of incubation on Potato Dextrose Agar plates. Based on morphological characterization, the assumed isolate FKF1 was *Xylaria* sp., FK511P was *Daldinia* sp., FK511H was *Penicillium* sp., and FK5112H was *Penicillium* sp. (Figure 3).

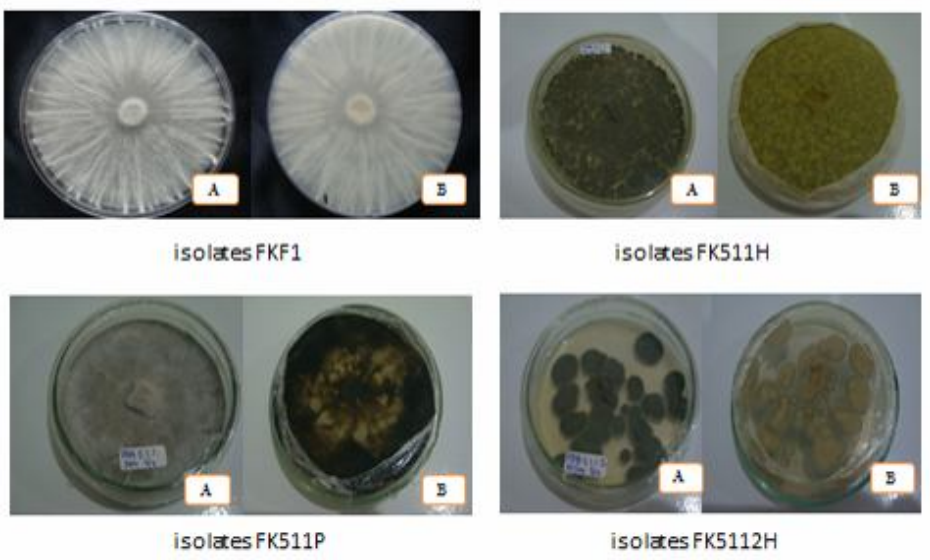

Figure 3. Morphological characteristics of endophytic fungi isolates: A - surface color, B - reverse color.

Molecular identification of fungi. Based on molecular identification using the Internal Transcribed Spacer (ITS) region, the DNA bands obtained were around 500-600 bp, the success of PCR was detected by the presence of a single DNA band around 600 bp; the PCR results can be seen in Figure 4.

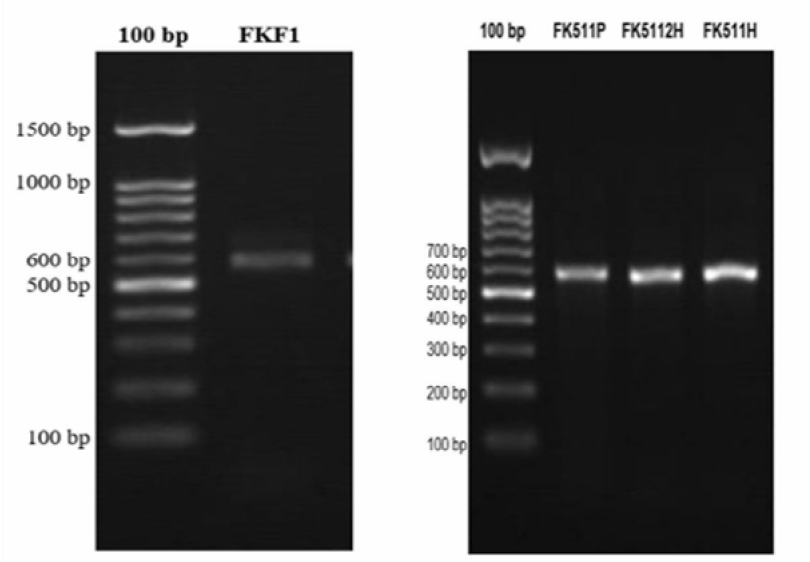

Figure 4. PCR product from FKF1, FK511P, FK5112H, FK511H.

The BLAST results in the GenBank of NCBI showed that the FKF1 isolate was similar to the fungus *Xylaria feejeensis*, FK511P isolate was similar to the fungus *Daldinia*

eschscholtzii, while FK511H and FK5112H isolates had similarities to the fungal *Penicillium citrinum*.

Table 1

Identification of endophytic fungi and closest relatives according to BLAST search

Morphologically identified-fungi	*Strain*	*Sequence length (bp)*	*Related strain (BLAST)*	*Accession number*	*Similarity (%)*
Xylaria sp.	FKF1	545	*Xylaria feejeensis*	MG871188.1	100.0
Daldinia sp.	FK511P	534	*Daldinia eschscholtzii*	MK334010.1	100.0
Penicillium sp.	FK511H	515	*Penicillium citrinum*	MN319561.1	100.0
Penicillium sp.	FK5112H	515	*Penicillium citrinum*	MN319561.1	100.0

The phylogenetic tree was created using Geneious v5.6 software with the Neighbor-Joining algorithm. In this phylogenetic tree, isolates FKF1 were grouped together with the fungal *Xylaria feejeensis* MG871188.1. FK511P isolates were grouped together with *Daldinia eschscholtzii* MK334010.1. FK511H isolates and FK5112H isolates were grouped together with *Penicillium citrinum* MN319561.1.

The three fungal isolates of *Xylaria, Daldinia* and *Penicillium* belonged to the Ascomycetes, the Xylariaceae family (*Xylaria, Daldinia*) and the Eurotiaceae family (*Penicillium*) (Alexopoulus & Mims 1979) (Figure 5). These fungal genera have an airborne and terrestrial origin and found endophytes in plants, mangroves, algae, seagrasses, sponges and cnidarians. It is possible that the fungal spores enter the marine environment by run-off from the land and made contact with gorgonian colonies (Preedanon et al 2016). The ascomycetes have been reported as endophytic fungi and as source of secondary metabolite compounds with antimicrobial activities (Bugni & Ireland 2004).

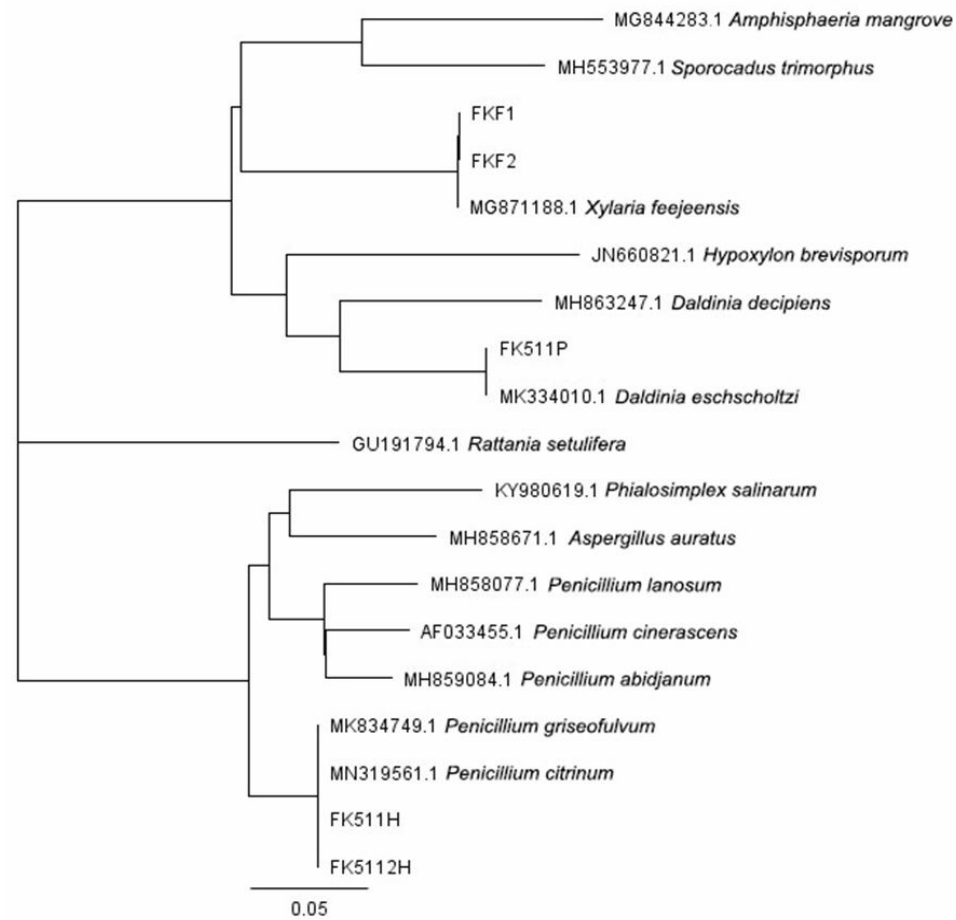

Figure 5. Phylogenetic tree isolates of endophytic fungi.

Antibacterial activities assay. The antibacterial activities of the extracts from fermentation broth and mycelia of three isolates endophytic fungi were assayed by using pathogenic bacteria *S. aureus,* MRSA, and *E. coli*. The ability inhibition activity of pathogenic bacteria can provide useful data to find promising antibacterial bioactive compound candidates.

The results of the inhibition zone of the endophytic fungi showed different levels of antibacterial activities with their broth and mycelia extract to pathogenic bacteria. Assay with pathogen *S. aureus* showed that the fungal *X. feejeensis* in the broth EtOAc extract with 15.0±1.0 mm inhibition and mycelia MeOH extract with 14.5±1.26 mm inhibition have the strong level. *D. eschscholtzii* in the broth EtOAc extract with 8.3±0.5 mm inhibition and mycelia MeOH extract with 8.1±1.04 mm inhibtion have the moderate level. *P. citrinum* in the broth EtOAc extract with 11.5±0.5 mm inhibition and mycelia MeOH extract with 12.3±0.58 mm inhibtion have the strong level. Assay with pathogen *E. coli* showed that the fungal *X. feejeensis* in the broth EtOAc extract with 8.5±0.50 mm inhibition has moderate level, and the mycelia MeOH extract with 14.5±1.32 mm inhibition has the strong level. *D. eschscholtzii* in the broth EtOAc extract with 9.0±0.50 mm inhibtion has moderate level and the mycelia MeOH extract with 12.1±1.61 mm inhibition has the strong level. *P. citrinum* in the broth EtOAc extract with 10.6±2.47 mm inhibition and the mycelia MeOH extract with 11.8±1.53 mm inhibition have a strong level. Assay with pathogen MRSA, the fungal *X. feejeensis* in the broth EtOAc extract with 13.3±1.15 mm inhibition and mycelia MeOH extract with 10.0±1.04 mm inhibition have the strong level. *D. eschscholtzii* in the broth EtOAc extract with 11.8±1.04 mm inhibition and mycelia MeOH extract with 17.1±1.15 mm inhibition have the strong level. *P. citrinum* in the broth EtOAc extract with 13±2.18 mm inhibition and mycelia MeOH extract with 14.5±0.87 mm inhibition have the strong level (Table 2).

Table 2

Antibacterial activities of the broth ethylacetat (EtOAc) extract and the mycelia metanol (MeOH) extract of endophytic fungi

Fungal		*Staphylococcus aureus*		*Escherichia coli*		*Methicillin Resistant Staphylococcus aureus*	
Class	*Species*	*Broth*	*Mycelia*	*Broth*	*Mycelia*	*Broth*	*Mycelia*
Ascomycetes	*Xylaria feejeenensis* FKF1	+++	+++	++	+++	+++	+++
Ascomycetes	*Daldinia eschscholtzii* FK511P	++	++	++	+++	+++	+++
Ascomycetes	*Penicillium citrinum* FK511H	+++	+++	+++	+++	+++	+++

Note: Inhibition diameters were used to define the categories of bacterial inhibition: +, inhibition diameter less than 5 mm (weak); ++, between 5-10 mm (moderate); +++, between 10-20 mm (strong); ++++, more than 20 mm (stronger) (Davis & Stout 1971). Assays were carried out in triplicates.

Based on the results, the fungal *X. feejeensis* FKF1, *D. eschscholzii* FK511P, and *P. citrinum* FK511H have a high inhibition against Gram positive bacteria *S. aureus,* MRSA, and Gram negative *E. coli*. It is possible that the inhibitory activity of these endophytic fungi come from the intracellular metabolite compounds or secrete extracellular bioactive compounds. According to Konings et al (1992), microbes can secrete a wide variety of metabolites including intracellular and extracellular products.

Previous finding of Rukachaisirikul et al (2009) showed a new compound [11]cytochalasin derivative, xylarisin, isolated from marine-derived fungus *Xylaria* sp. PSU-F100 from gorgonian *Annella* sp., together with six known metabolites, three mellein derivatives: (R)-(-) mellein methyl ether, (R)-(-)-5-carboxymellein, and (R)-(-)-5-hydroxymethylmellein, one pyrone derivative: 6-[(1R)-1-hydroxypentyl]-4-methoxy-2H-pyran-2-one, and two carboxylic acids: (2E, 4S)-2,4-dimethyloct2-enoic acid and piliformic acid. All isolated compounds showed antibacterial activity against *S. aureus* ATCC 25923 and MRSA. Preedanon et al (2016) also found the endophytic fungi *Xylaria* sp. from *Annella* sp. from Thailand waters which has antibacterial activity against *S. aureus,* MRSA, *E. coli, Pseudomonas aeruginosa*, and antifungal activity against *Candida*

albicans, Cryptococcus neoformans and *Microsporum gypseum*. Guo et al (2019) reported two new compounds of hexaketides, xylarodons B and C isolated from the endophytic fungi *Xylaria* sp. SC1440, the compounds were evaluated for tyrosinase inhibitory activity and its cytotoxicity. Espada et al (1997) reported that the fungal *Xylaria* sp. produces secondary metabolites, mainly from the class of cytochalasins, isocumarins, xanthones, lactones, triterpenes, sesquiterpenes, which have various biological activities, such as antimicrobial, antioxidant, antibiotic, anti-inflammatory, anticancer and anti-tumor properties.

Hu et al (2014) reported five new compounds from marine-associated fungi ethylacetate (EtOAc) extract, *Daldinia eschscholzii* isolated from *Scaevola sericea* Vahl, from mangrove forests in Haikou, China. The five new compounds include a benzopyran ribonic glycoside, daldiniside A, two isocoumarin ribonic glycosides, daldinisides B and C, and two alkaloids, 1-(3-indolyl)-2*R*,3-dihydroxypropan-1-one and 3-ethyl-2, 5-pyrazinedipropanoic acid. The number of studies with *Daldinia* spp. have revealed more than 20 new bioactive metabolites, including derivatives of benzofenones (Hashimoto et al 1994a), azafilones, daldinins A-C (Hashimoto et al 1994b), cytochalasans (Buchanan et al 1995; 1996a, 1996b; Hashimoto & Asakawa 1998), triterpenoids, concentricols (Stadler et al 2001; Quang et al 2002a; 2002b), daldiniapyrone, daldinones (Quang et al 2002b), benzoquinones (Qin & Liu 2004a), esteroids (Qin & Liu 2004b), heptentriols (Wang & Liu 2004), diaporthins, orthosporins (Lee et al 2006), and concentricolides (Qin et al 2006). Dalesconol A and B polyketides with immunosuppressive activity were initially isolated from *Daldinia eschscholtzii* by Zhang et al (2008). Further, daeschol A, dalesconol C, 2, 16-dihydroxyl-benzo[j]fluoranthene and dalmanol A were isolated for the first time from mantis-associated *Daldinia eschscholtzii* (Zhang et al 2011). Recently, helicascolide C, a new lactone with fungistatic activity against *Cladosporium cucumerinum* was isolated together with helicascolide A from an Indonesian marine algicolous-associated *Daldinia eschscholtzii* (Tarman et al 2012).

Khamthong et al (2012) obtained penicillanthranins A isolated from the sea fan-derived fungi *P. citrinum* PSU-F51 that displayed moderate antibacterial activity against *S. aureus*. Kozlovsky et al (2000) reported a new fungal metabolite from *P. citrinum,* cyclocitrinol. Sasaki et al (2005) obtained a novel tetracyclic alkaloid, perinadine A, isolated from the broth of *P. citrinum* collected from the gastrointestinal of a marine fish. Tsuda et al (2004) found a novel pentacyclic alkaloid, citrinadin A, isolated from the broth of *P. citrinum* separated from a marine red alga. Tsuda et al (2005) reported three new pyrrolidine alkaloids, scalusamides A-C, isolated from the broth of *P. citrinum*, separated from the gastrointestinal of a marine fish, scalusamide A, exhibiting antifungal and antibacterial activities. Kozlovsky et al (2003) reported quinocitrinines A and B, new quinoline alkaloids from *P. citrinum*, a permafrost fungus.

Most studies reported that metabolites of marine endophytic fungi inhibited Gram-positive bacteria (Christophersen et al 1999; Zainuddin et al 2010; Wang et al 2011). However, from this results the fungal *X. feejeensis* FKF1, *D. eschscholzii* FK511P, and *P. citrinum* FK511H isolated from sea fan *Annella* sp. have a high inhibition against Gram positive and Gram negative bacteria. These three marine-endophytic fungi were expected to be new potential producers of new antibiotic compounds that have the broad spectrum. Preedanon et al (2016) also obtained many novel antimicrobial and bioactive compounds isolated from sea fan-endophytic fungi from Thailand, so that this report confirms that sea fan-endophytic fungi are a source of antibacterial and new compounds.

Conclusions. The three endophytic fungi isolated from sea fan *Annella* sp. from Bunaken waters, North Sulawesi showed potential antibacterial inhibition againts several pathogenic bacteria, Gram positive *Staphylococcus aureus,* Methicillin Resistant *Staphylococcus aureus* (MRSA), and Gram negative *Escherichia coli.* Molecular characterization indicated that endophytic fungi were *Xylaria feejeensis, Daldinia eschscholtzii* and *Penicillium citrinum.* Further research needs to determine the bioactive compound which has a role in inhibiting the growth of the pathogenic microbes so that this result could be one of the sources for new antibiotics compound discovery.

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