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Isolation and Identification of Sponge-Associated Fungus Producing Anti Multidrug-resistant (MDR) Bacterial Substances.

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ABSTRACT

Marine sponges are rich sources of bioactive substances with various pharmacological activities. Previous studies have shown that most bioactive compounds wereoriginally produced by associated-microorganisms. In our study on marine pharmaceuticals, we collected 19 sponge specimens from Riung water, Nusa Tenggara Timur, Indonesia. A total 33 fungi isolates were isolated from the sponges. Then, the isolates were screened for antibacterial activity using overlay method against the MRSA and MDR *E. coli.*Then, the active isolates were cultured on malt extract broth medium to provide the crude extract that tested against the MRSA and *E. Coli* using. One isolate, anassociated of the sponge *Agelas* sp. showed to be amostpromising fungus.The isolate was determined based on morphological characteristics and genetic analyses as *Fusarium solani.* The extract of the isolate has astronger activity to *E. coli* and *S. aureus* than the activity of the chloramphenicol. It can be concluded that the isolate has potential as a new source of antibacterial compounds.

Keywords.*Fungus, Fusariumsolani, Staphylococcus aureus, Escherichia coli,* Sponges

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INTRODUCTION

Marine sponges have been shown to be a rich source of antibacterial compounds with a potential for pharmaceutical applications. Naturally, the compounds have ecological properties for sponge survival, such as repelling predators[1], depressing competitors[2], and disinfecting pathogens[3]. Some of the compounds showed pharmacological potency as antitumor[4], antibacterial[5], antifungal[6], andantiinflammatory[7], antimalarial[8]. However, only a few of the bioactive compounds passed to the pre-clinical and clinical testing stage[9],as they are highly sourced limited[10]. Collecting a large amount of a particular compound from nature will inevitably disturb the local ecology. Chemical synthetics are the preferred method for producing the bioactive compounds, however, in many cases, this method is not feasible due to the complexity and chirality of the target compounds[11].

Sponges are well known to contain a lot of microorganisms within the mesophyll. In some cases, bacteria contribute up to 40% of the sponge biomass and appear to be permanently associated with the host sponges[12].It ispresumed that microorganisms play an important role, such as providing food, bioactive compounds, or their precursors to the host[13].Several studies indicated that some metabolites were produced by associated microorganisms[14].

In this paper, we describe the isolation and identification of the sponge-associated fungus producing anti-MDR bacteria from a marine sponge collected from Riung Water, East Nusa Tenggara, Indonesia.

MATERIALS AND METHODS

Sponge sample collection: The marine sponges were collected from the TujuhBelasPulau, Riung, East Nusa Tenggara, Indonesia by SCUBA divingat 3-15 m depth (Figure 1).The specimens were kept cool until the inoculation process[15].

Figure 1. Collection site of the marine sponges KN-19 in Riung, East Nusa Tenggara, Indonesia (Map Soure: Google Map).

Isolation of the associate fungus: Isolation of the fungi from the sponges was conducted using the method proposed by Subramani *et al.* (2013)[16] with modifying on the sponge size and media. The sponges were washed with sterilized sea water before inoculation process to remove any associated microorganisms from their surface. Each specimen was then cut into small pieces (approx. 1 cm x 1 cm x 0.3 cm). The fungus was isolated by putting each piece of each sponge directly on the surface of malt extract agar (MEA) medium; one agar plate for each sample to avoid contamination and confusion regarding the source of fungus. After incubation for three days, the fungus colonies were separated based on their morphological characteristics, and each colony was inoculated on a new agar plate containing MEA.This process was repeated until a pure sample of each isolate was obtained.

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Screening for antagonistic potential: Initial screening was conducted using the overlay method from[17].The assay was conducted on an MEA medium against the multidrug-resistant(MDR)*Staphylococcus aureus* and *Escherichia coli* isolated from Karyadi Hospital, Semarang, Central Java, Indonesia. The *S. aureus*is resistant to several commercial antibiotics such as kanamycin, gentamycin, amikacin, ampicillin, oxacillin, imipenem, penicillin, amoxicillin clavulanic acid, erythromycin, ceftriaxone, and ciprofloxacin. The *E. coli*is resistant to ampicillin, penicillin, amoxicillin clavulanic acid, erythromycin, ceftriaxone, trimethoprim/sulfamethoksazol, vancomycin, ciprofloxacin, and nalidixic acid.

The fungal isolates were grown on MEA and incubated for 24 hours. The antagonistic bacteria *S. aureus* and *E. coli* were grown in broth ZoBell 2216E medium.After a 24-hoursincubation period, 1 ml of each antagonistic bacteria suspension were diluted in 99 ml of *soft agar*ZoBell 2216E. The *soft agar* was poured onto the MEA medium containing the fungal isolates and incubated for 24 to 48 hours depending on the fungus species. The inhibition zone indicated that isolates were active against the antagonistic bacteria (the data are not shown in this paper).

Sponge Identification: The sponge host of the isolates producing anti-MDR bacteria was identified based on morphological and spicules characteristics according to a method proposed by [18].The features were examined for morphological characteristics including colony shape, color, texture, consistency, and surface type. For the spicules preparations, a small fragment of the sponge was placed in a small Erlenmeyer flask, then a small quantity of commercial bleach (sodium hypochlorite) was added. After the organic components dissolved leaving only the mineral skeleton, the bleach was diluted with ethanol and removed carefully. The washing process was repeated until the clean spicules were obtained. The clean spicules suspension was transferred onto a glass slide and observed and photographed at 100x and 400x magnification using a microscope equipped with a digital camera.

Extraction of the cultured isolate: The pure isolatethat active against the *S. aureus* and *E. coli* werecultured in 1 L malt extract broth (MEB) medium for seven days or until the maximum growth at ambient temperature. The medium was filtered to obtain the fungal mycelium andwas then extracted with methanol at room temperature. The solvent was filtered using Whatman filter paper, and the filtrate was concentrated using arotary evaporator under a vacuum to obtain the methanolic extract [19].

Bioassay of the methanolic extracts: The extracts were testedfor the *S. aureus* and *E. coli* using the disk diffusion agar method at concentrations of 400, 200, 100, 50 and 25 µg/disk with triplicates. The antagonistic bacteria were inoculated on MEA medium and incubated for two hours before setting the paper disks containing the methanolic extract on the medium agar surface. The inhibition zones were measured after 24 and 48 hours as an indicator for the activity of the extracts.

Characterization of potential KN19-1 isolate

Phenotypic characterization: The potential isolate was grown on MEA medium to examine and photograph the morphological characteristics under a microscope. Isolate identification was conducted using a fungal taxonomical book and taxonomical guidance [20].

Molecular identification of the fungal using 18s rRNA gene analysis: The DNA isolate was extracted with *Chelex*following the instruction provided by the company (Bio-Rad)[21] (Walsh*et al.,* 1991).

In brief, the mycelia were added with 50 µL-100 µL aquabides (ddh2O)and 1 mL of diluted saponin in PBS. The mixture was incubated for 24 hours in 4 °C before centrifugation at 13,000 rpm for 1 minute for supernatant separation. A 250 µl of 10% chelex was added to the DNA extract and homogenized under vortex for 20 secondsbefore centrifugation at 13,000 rpm for 2 minutes. Then, the mixture was heated at 95°C for 45 minutes and homogenized using vortex. Finally, the DNA extract of the isolate was obtained after removing the supernatant post centrifugation at 13,000 rpm for 2 minutes.

The DNA in the supernatant was extracted with isopropanol for further analyses with electrophoresis on the agarose gel. Electrophoresis was run at 100 volts using TAE(Tris-Acetate-EDTA) as a running buffer. Gels were observed under UV light after strained with ethidium bromide.Amplification PCR was conducted using

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universal primer for 18s rRNA and sequenced on sequencer *BigDye Applied Biosystem* using the method described in by Handayono and Rudiretna (2000)[22]. Sequencing was conducted in MACROGEN Korea.

Analysis of sequence alignment of 18S rRNA gene and phylogenetic analysis: Phylogenetic analysiswas conducted using BLASTn software provided by Gen Bank (NCBI), followed by alignment process using Clustal X software. The phylogenetic tree was developed using a MEGA version5 software.

RESULTS AND DISCUSSION

From Riung Water of Nusa Tenggara Timur Province-Indonesia (Figure 1.), a total of 19 sponges were collected that provided 33 fungal isolates. After antagonistic bioassay, the most active isolate, KN19-1,was chosen for further study[23]. The host sponge of the isolate was also identified base on morphological characteristics and spicules analyses.

The sponge KN19-1 had anorange fan shaped with a rounded or lobate edge. The sponge was compressible and tough (difficult to tear) with a porous "body" (Figure 2a and 2b).This species was found in coral reef ecosystems, usually attached to the hard substrate. Microscopic investigation of the spicules observed the present of oxea and style(Figure 2c).Oxea and style are very common in the order of Agelasida sponge[24]. Evaluation of the morphological characteristics and spicules type depicted that the sponge KN19-1 belonged to genus *Agelas.* This conclusion also agrees with Hooper [18] who noted that sponge of Agelasidahasfan-shaped, well developed spongin-fiber skeleton, with regular and irregular reticulation, fibers echinated by short styles or oxeas with verticillate spines; microscleres absent. Furthermore, the Agelasidae has lamellate characteristics, tubular or massive, often "honeycomb" reticulate inconstruction; color frequently orange or red, texture extremely tough but compressible, reflecting the highratio of sponging-fiber to spicule.

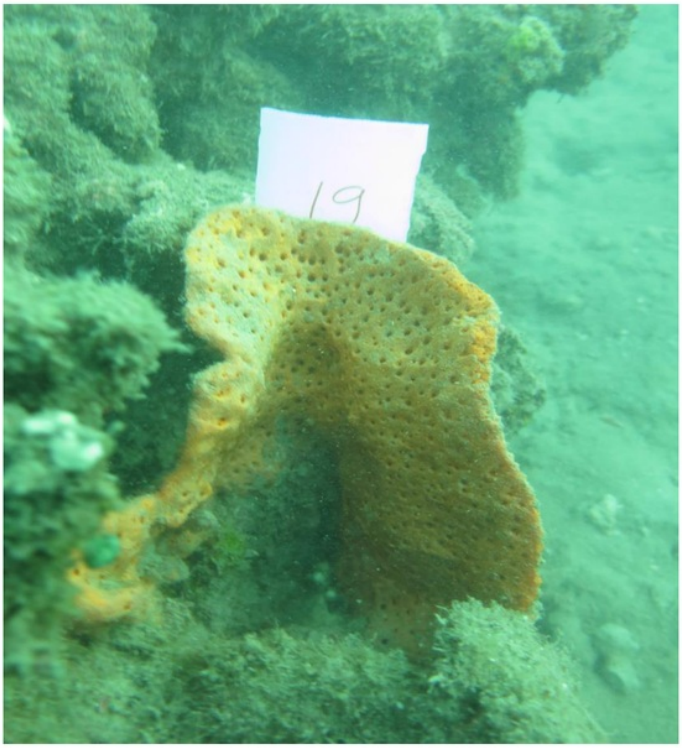

Figure 2 (a) In situ picture

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Figure 2 (b): In the laboratory picture

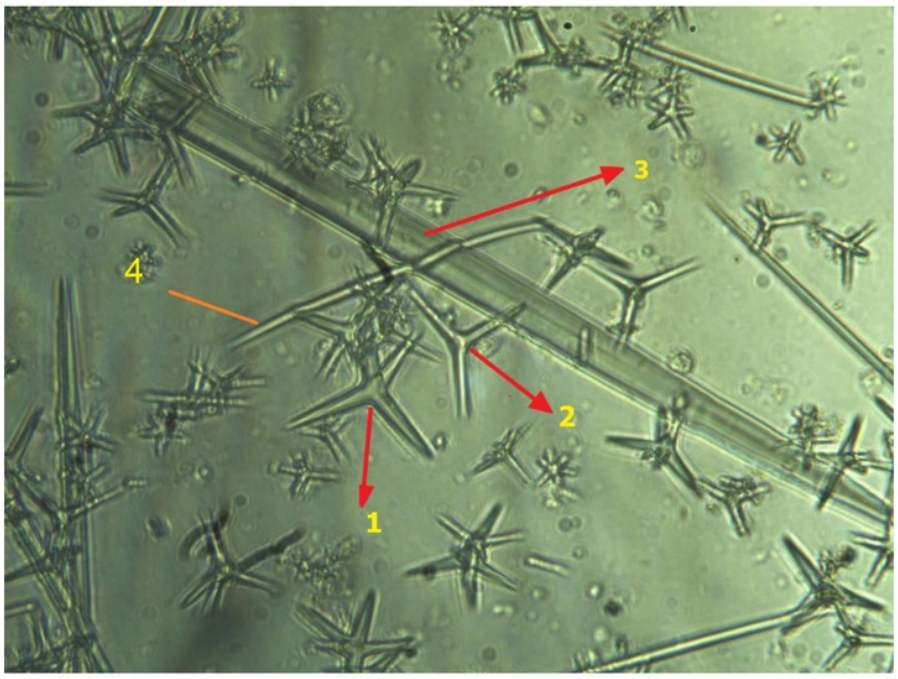

figure 2 (c) 1. calthrop, 2. triod, 3. style

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Sponges of the genus *Agelas*are a rich source of bioactive compounds. Among the compounds has various bioactivities, such as immune stimulantenzyme inhibitor[25], anti-fouling[26], and antimicrobial[27].

On MEA medium, the isolate had awhite colony with whitish mycelium. Macroconidia were three to four septa on average and were slightly curved. Microconidia were abundant and canoe-shaped (Figure 3).The genomic DNA from KN19-1 isolate was amplified by PCR. This amplified gene product was used for identifying the fungal isolate. The consensus sequence of 560bp of 18S rRNA gene was generated from forward and reverse sequence data and searched through the BLAST algorithms of the NCBI website. The BLAST results revealed that the isolate KN19-1 showed 99% similarity with various isolates of *Fusarium solani* (GenBank accession no. HQ651165.1). The phylogenetic relationship of the KN19-1 isolate was also established with its 22 nearest neighbors(Figure 4) and the nearest identified fungal isolate with which its closest relationship was established belonged to *F. solani* isolateFSNGR147(GenBank accession no. HQ651165.1). Hence, this KN19-1 isolate could be concluded as anisolate of *F. solani*. At present, the isolate was deposited in the GenBank with accession numberLC091211.

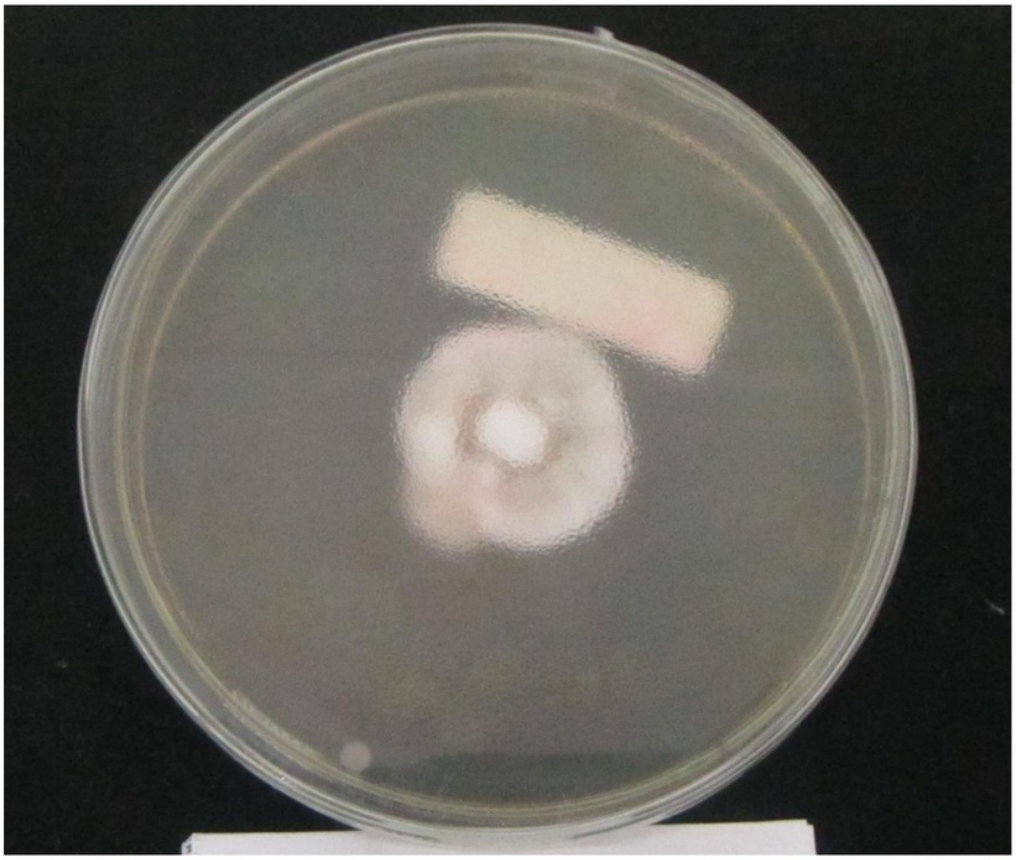

Figure 3 (a): The fungus colony on MEA medium

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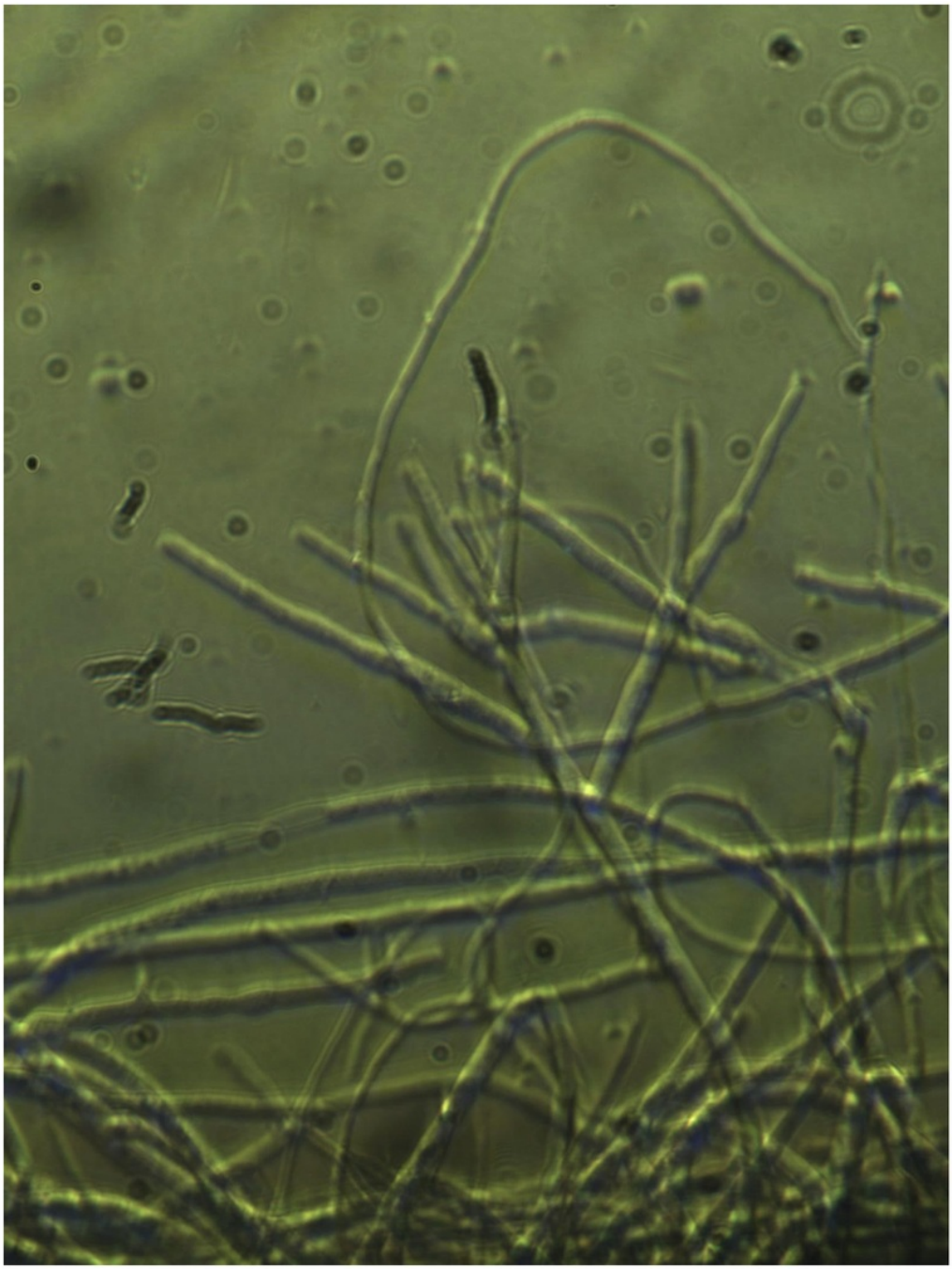

Figure 3 (b): The microscope picture

No.	Isolate code	Nucleotide length	Genetic relationship (%)	Homology (%)	Access No (Blast NCBI)
1	KN19-1	511	*Fusarium solani* Isolate FSNGR147	99 %	HQ651165.1

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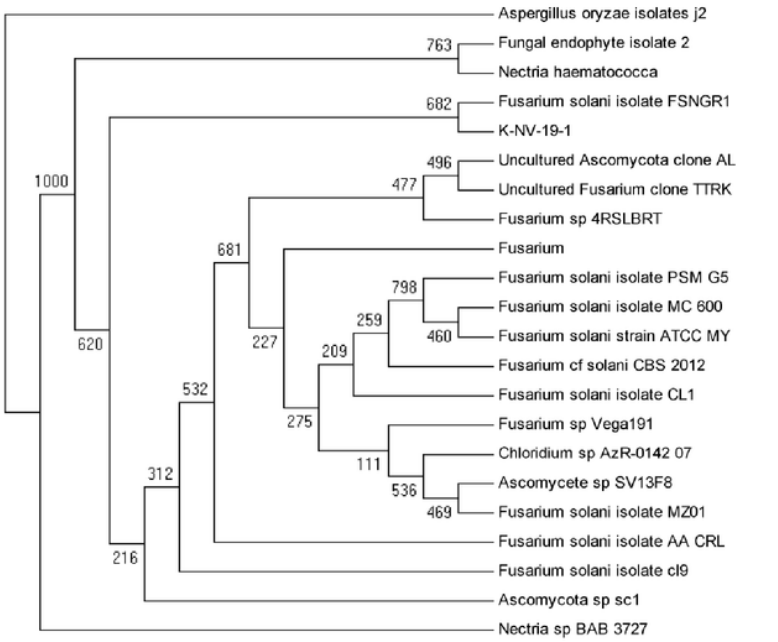

Figure 4: Phylogenetic tree fungus KN-19 ClustalX

The genus *Fusarium*isascomycetous filamentous fungus causing diseases on animals and plants, toxin producers, and debris decomposers.*Fusarium solani* is a very common soil fungus and well known as a causal pathogen of many plant diseases, some of which have significant economic and ecological impacts. This fungus causes damping-off and root rot in seedling nurseries, the poor establishment of out-planted seedlings and root rot of mature plants[28].*Fusarium solani* has been known to be a very persistent pathogen in the soil as it can formchlamydospores, and capable of surviving in infested soils for a long period[29]. The fungus can also survive as mycelium or spores in infected or dead tissues. The symptoms of the disease are indicated by black discolorations of the main root and lateral roots, beginning at or near the soil level. The above ground symptoms include a yellowing of the leaves from the lowest part of the plant, and many infected plants do not produce pods. The Fusarium also infectsplant and seed that can failtransplanted seedling to become established [30].

In antagonistic tests, the isolate KN19-1 showed active against the both MDR bacteria, *S.aureus* and *E. coli.* The methanolic extract of the isolate also exhibited strong activity against both pathogens with inhibition zone 17.36±0.188 (*S. aureus*)and 7.35 ± 0.070 (*E. coli*)at the concentration 400 µg/disk that stronger activity where the control (chloramphenicol 100µg/disk) has inhibitionzone against *S. aureus* and *E. coli*8.94 mm and 8.52 mm, respectively.Terrestrial plant endophytic *Fusarium* sp. was reported to be active against the MRSA with MIC 16 mg/mL[31]. Another research reported that Fusarium sp isolated from the soil did not activeagainst the MRSA [32].*Fusarium tricinctum*produced bromomethylchlamydosporols A and B showed activity against *S. aureus* (MIC, 15.6 µg/mL) [33]. Therefore, the isolate can be developed as a source of thenewanti-MDR microbial drug. Our future project will be on searching of large scale production methods using the most economical material.

CONCLUSION

Fusariumsolani, a terrestrial origin fungus, was found associated with the sponge *Agelas* sp. The isolate has the potential as a new source of compounds with anti-MDR*S. Aureus* and *E. coli* properties.

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