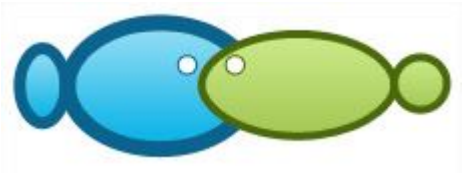

Anti MDR *Acinetobacter baumannii* of the sponges-associated fungi from Karimunjawa National Park

[1,2,3]Aninditia Sabdaningsih, [1,3]Olvi Cristianawati, [4]Mada T. Sibero, [3]Muslihuddin Aini, [4,5]Ocky K. Radjasa, [4]Agus Sabdono, [3,4]Agus Trianto

[1] Doctoral Program of Coastal Resources Management, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia; [2] Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia; [3] Marine Natural Product Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia; [4] Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia; [5] Ministry of Research, Technology and Higher Education of the Republic of Indonesia, 10270 Jakarta, Indonesia. Corresponding author: A. Sabdaningsih, aninditiasabdaningsih@live.undip.ac.id

Abstract. Marine environment is an enormous source of bioactive compounds with potential as a pharmaceutical agents. Such organisms that produce potential bioactive compounds are sponges, which are filter feeders with many associated microorganisms. Sponges-associated fungi have been reported as an antibacterial sources. This study aimed to obtain and to identify the potential sponges-associated fungi that are active against mulltidrug resistance (MDR) *Acinetobacter baumannii.* The sponges were collected from Karimunjawa Islands. The MDR *A. baumannii* was obtained from Kariadi hospital that is resistant against at least 11 antibiotics. The fungal symbionts were isolated by tapping method followed by colony isolation based on the morphological characteristics. Antibacterial assay was conducted by disk diffusion agar. The potential fungal isolates were cultured on malt extract broth (MEB). The potential isolate was identified based on the molecular analysis. The crude extract of KJ-S03.1 was characterized with Thin Layer Chromatography and phytochemical test. A total of 24 fungi were isolated from ten sponges. Among the isolates, the fungus KJ-S03.1 showed the most potential based on the activity against MDR *A. baumannii.* The molecular analysis identified that the fungus KJ-S03.1 is *Aspergillus nomius.* The sequence was deposited into Genbank with accession number LC415575.1. The extract of the isolate contains alkaloid, quinone and flavonoid. Isolate *A. nomius* KJ-S03.1 is a potential source of antibacterial agent that is active against the MDR *A. baumannii.*

Key Words: *Acinetobacter baumannii, Aspergiillus nomius,* Karimunjawa, marine fungi, MDR, sponges.

Introduction. *Acinetobacter baumannii* is an opportunistic bacterium that is commonly reported causing nosocomial infection in immune-compromised patients (Howard et al 2012). In several cases, this pathogen also has been reported as the causative agent of bacteremia, pneumonia / ventilator-associated pneumonia (VAP), meningitis, urinary tract infection, venous catheter infections and wound infection (Maragakis & Perl 2008; Fishbain & Peleg 2010; Michalopoulos & Falagas 2010; Howard et al 2012). The treatment of *A. baumannii* infection is more rigid since the occurrence of drug-resistance in this pathogen. The drug-resistance of *A. baumannii* has been reported for the past 30 years (Almasaudi 2018). Multidrug-resistant (MDR) *A. baumannii* was reported causing nosocomial and blood stream infections in several regions in Indonesia (Tjoa et al 2013; Primaningtyas et al 2017; Nirwati et al 2018; Saharman et al 2018). Furthermore, the outbreaks of drug-resistant *A. baumannii* occured in several countries such as China (Hujer et al 2017), Italy (Venditti et al 2019), Spain (Valencia et al 2009), Thailand (Molter et al 2016), Turkey (Atik et al 2018) and US (Yagnik et al 2019). These reports

show that multidrug-resistant *A. baumannii* is not only Indonesia's health problem but also a global health issue. Therefore, exploration of new antibiotic to cure the *A. baumannii* infections is needed.

Indonesia as a tropical country is a harbor of plenty of bioactive compounds that are potentially used for a new candidate of antibiotics which were produced by the marine organisms (Hamdillah et al 2019; Hanif et al 2019; Sibero et al 2019a). In order to obtain new antibiotics, exploration of marine natural products revealed sponge as the most prolific marine organisms as bioactive producer (Sabdono & Radjasa 2008; Trianto et al 2011; Trianto et al 2014; Mehbub et al 2014; Hu et al 2015; Hanif et al 2019). Several antimicrobial compounds were isolated from marine sponge such as illimaquinone, langcoquinones A-B, langcoquinones D-F, smenospongine, and tribromoiododiphenyl ether (Nguyen et al 2018; Ki et al 2018; Ito et al 2018; Balansa et al 2019). Unfortunately, this practice might damage to the marine environment. Therefore, exploration of antimicrobial compounds sponge associated-microorganisms has been promoted.

In 2016, a total of 272 sponge-associated microorganisms were reported as a potential producer of antimicrobial compounds (Indraningrat et al 2016). Among all reported microorganisms, fungi only possessed 10%. Interestingly, our previous studies reported the ability of sponge-associated fungi inhibiting the MDR bacteria such as ESBL *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* (MRSA), and *Salmonella typhi* (Sibero et al 2017a; Trianto et al 2017; Sibero et al 2018a; Sibero et al 2018b; Sibero et al 2019b; Sibero et al 2019c). However, none of the studies reported the ability of sponge-associated fungi inhibited MDR *A. baumannii*. Moreover, the discovery of antibacterial compounds against *A. baumannii* from sponge-associated fungi is still rarely conducted. This research was designed to isolate sponge-associated fungi producing the anti-MDR *A. baumannii* substances, to identify the most potential isolate, and to characterize its crude extract.

Material and Method

Sample collection. Sponges were collected from the Karimunjawa National Park, Jepara, Central Java, Indonesia (5°80'31.60"S and 110°50'8"E) at 5-10 m depth by SCUBA diving on March 2017 (Figure 1). The specimens taken were about 3-5 cm^2 by purposive random sampling. Specimens were kept in Ziploc plastic bag to be stored temporarily in a cool box and isolated directly to avoid contaminations.

Isolation and purification of sponges-associated fungi. Isolation of sponges-associated fungi was conducted using published protocols by Kjer et al (2010) and Sibero et al (2018b). Sponges were initially sprayed with sterile seawater and cut into approximately 1 cm^2 pieces in order to take fungi out from the tissue. Samples were incubated at room temperature with two replications in Malt Extract Agar (MEA) media (30 g malt extract, 5 g mycological peptone, 15 g agar, in 1000 mL sterilized natural sea water). The chloramphenicol was added to the media with a concentration 10 µg L^{-1} to inhibit the bacterial growth. Incubation time was within three until seven days depending on the mycelial growth. The fungal isolates were purified based on the morphological colony characteristics such as colour in reverse and front side, margin, and elevation. (Sabdaningsih et al 2017).

Multidrug resistant test of Acinetobacter baumannii. A clinical *A. baumannii* strain MDR was obtained from Clinical Microbiology Laboratory of Kariadi Hospital Semarang, Indonesia. Bacterium was directly isolated from patient. Then, the test was conducted using Biomerieux Vitek-2®. The first step was suspensions preparation. The suspensions were made by swabbing pure culture into 3 mL saline water in a test tube. The turbidity was checked using a DensiCheck™ about 0.50-0.63. The microorganism in a test tube was placed into a special rack (cassette) and the identification card was placed in the neighbouring slot. The filled cassette then was transferred into a vacuum chamber station. The process was running. Afterward the inoculated cards were passed by a

mechanism, then loading into the carousel incubator. All card types are incubated online at 35.5°C within some hours.

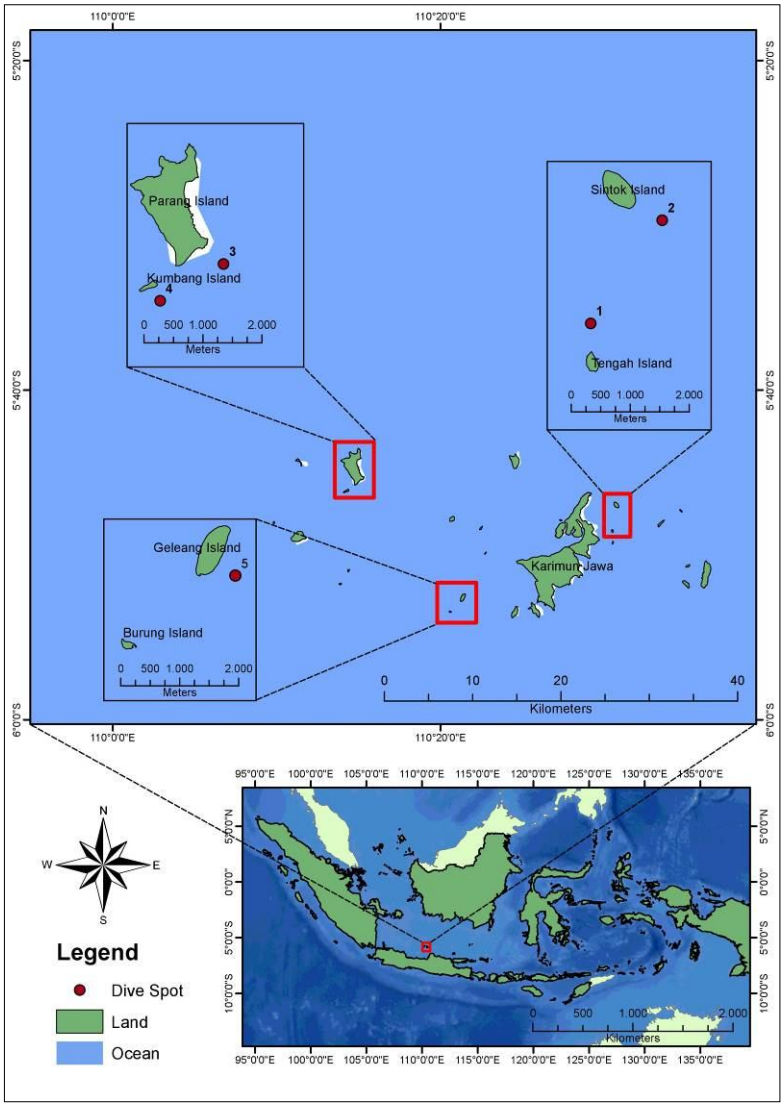

Figure 1. Sampling sites (1-5) of the marine sponges in Karimunjawa National Park on March 2017 were indicated in red dots. Map source: Institution of Geospatial Information of The Republic of Indonesia, 2017.

Antibacterial preliminary test. The agar plug diffusion method (Balouiri et al 2016; Sibero et al 2017b) was employed to screen potential isolates. Sponge-associated fungi were grown in marine MEA for 7-14 days. Mueller Hinton Agar (MHA) (300 g beef-dehydrated infusion form, 17.5 g casein hydrolysate, 1.5 g starch, 17 g agar, and 1000 mL sterilized distilled water) was prepared for a preliminary test of antibacterial activity. MDR *A. baumannii* clinical isolate with density 0.5 McFarland were inoculated onto the surface of MHA. Then, sponge-associated fungi were cut into round shapes and afterward transferred onto MHA. The plate was incubated at room temperature for 1 x 24 hours. The presence of an inhibition zone indicated the production of antibacterial compounds. Fungi that showed the widest inhibition zones were used for subsequent steps.

Secondary metabolite extraction from the potential isolate. Extraction was initiated by growing the potential fungus from the preliminary test in Malt Extract Broth (MEB) media (17 g malt extract, 3 g mycological peptone, in 1000 mL sterilized natural sea water) for 14 days at room temperature. The inoculum was separated from mycelia and medium with filter paper. Ethyl acetate (EtOAc) was added to the media at a 3:1

ratio. The medium with solvent was then separated with a separatory funnel, afterward, samples were evaporated with a rotary evaporator (Sabdaningsih et al 2017).

Antibacterial susceptibility test. Crude extract (10 µg μL^{-1}) from the potential isolate was prepared for the antibacterial susceptibility test against MDR *A. baumannii*. An inoculum of MDR test bacteria with a density 0.5 McFarland was swabbed onto Mueller-Hinton Agar (MHA). This method refers to Balouiri et al (2016). Crude extracts were added to the blank paper disc with repetition and subsequently incubated for 1 x 24 hours at room temperature. The clear zone was measured by using caliper (Ayuningrum et al 2019).

Sponge identification of the potential isolate. The sponge samples were preserved in ethanol, and dissected in small size pieces. The slice of this section was observed in slide glass using Microscope from low to high magnification. The spicules observation was prepared with soaking a piece of sample to sodium hypochlorite solution in five minutes. A few mixture solution was transferred using pippete to slide glass and a few of the distilled water was added to remove debris with spicules. The spicules were observed under microscope (van Soest et al 2014).

Molecular identification of the potential isolate. DNA extraction was conducted using *Chelex* suspensions (Walsh et al 1991). Amplification was performed using PCR with primers targeting the ITS region (18S rRNA gene), ITS1 as the forward primer with the sequence TCCGTAGGTGAACCTGCGG and ITS4 as reverse primer with the sequence TCCTCCGCTTATTGATATGC. The cycling program consisted of initial denaturation at 95°C for 3 minutes, followed by 35 cycles (denaturation at 95°C for 3 minutes, annealing at 52°C for 45 seconds, and extension at 72°C for 1 minute), then post cycling in 72°C extension for 7 minutes and a final hold at 16°C (Sibero et al 2017a). Furthermore, gel electrophoresis was conducted to visualize the DNA bands formed using a Gel Doc System. The PCR product was sequenced by 1st Base, while the phylogenetic tree was constructed using MEGA X. The sequence was then compared with other species with antibacterial properties in the phylogenetic tree using the Maximum Likelihood that combine with the Neighbor-Joining method, combine to bootstrap analysis setting with 1000 replications.

Metabolite characterization. This step was conducted to characterize the substances in the potential crude extract. The crude extract was fractioned using Thin Layer Chromatography (TLC) with dichloromethane: ethyl acetate: methanol (6:4:1, v/v) as the mobile phase and Si-60 as the stationary phase. Sample was transferred into Si-60 with capillary pipe. The result was visualized with a 356 nm UV lamp. Sample then was tested for phytochemicals to find out the group of compounds that contained in crude extract (Deshmukh & Theng 2018; Sibero et al 2019c).

Results and Discussion

Sponges collection. A total of 13 sponges were collected from Karimunjawa National Park, Jepara, Central Java, Indonesia. Three specimens were collected from site 1, encoded KJ-S03, KJ-S18, and KJ-S16. Two samples were collected from site 2, encoded KJ-T17 and KJ-N16. Five samples were collected from site 3, encoded KJ-N21, KJ-N23, KJ-S26, KJ-S27, and KJ-S30. Two samples were collected from site 4, encoded KJ-S102 and KJ-N25. The last sample encoded KJ-S47, was collected from site 5. They were included in genera, such as *Carteriospongia (*syn. *Phyllospongia*) sp. KJ-S03, KJ.S.30, *Dysidea* sp. KJ-N21, *Aaptos* sp. KJ-N25, KJ-S47, *Callyspongia* sp. KJ-T17, *Cynachirella* sp. KJ-S18, *Clathria* sp. KJ-S16, KJ-S26 and *Liosina* sp. KJ-N25. The morphology of samples in the waters is depicted in Figure 2.

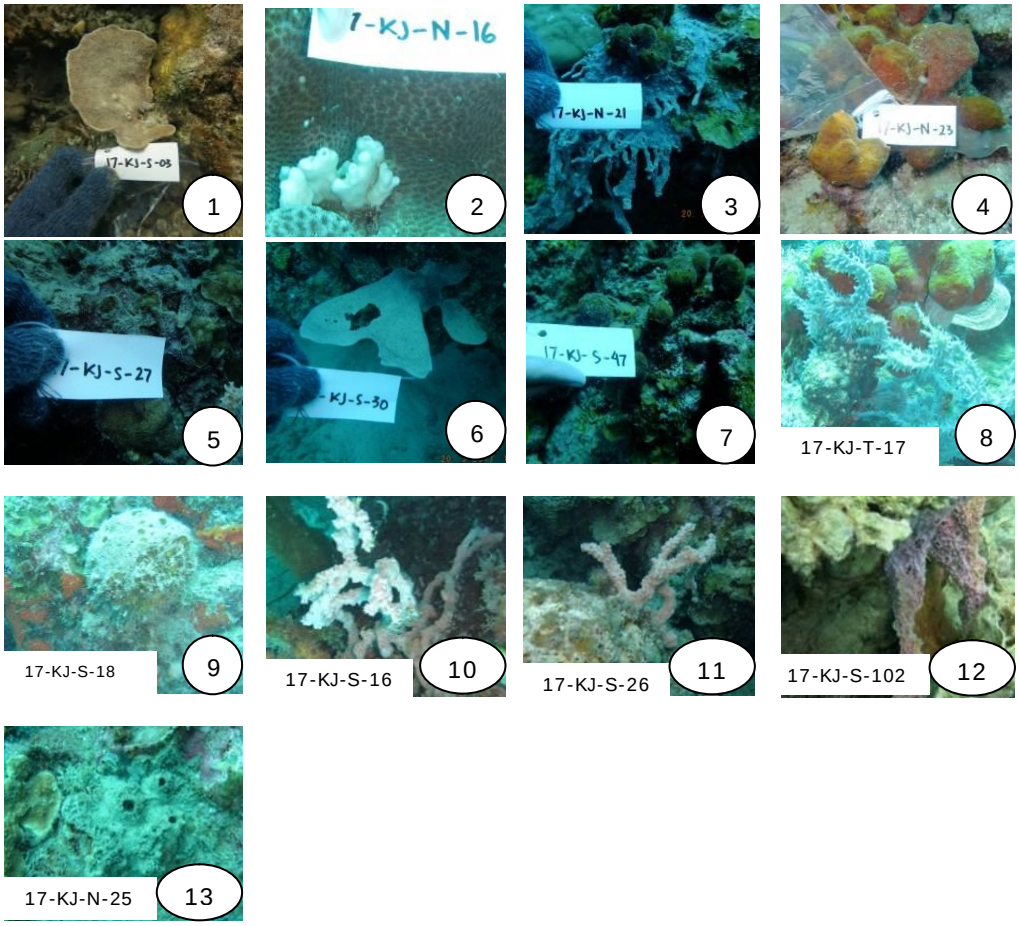

Figure 2. Underwater photographs of the collected sponges (1-13) from Karimunjawa National Park.

All of those samples have been reported for their ability as a pharmaceutical agent. For instance, *Carteriospongia* (syn. *Phyllospongia*) sp., have been studied for 37 years. This genus of sponge has a numerous compounds with various activities such as antimacroalgal activity, cytotoxic, anti-thrombocyte (Zhang et al 2017; Kasmiati et al 2018). Aaptamine derivatives isolated from *Aaptos aaptos*, showed antifungal activity againts *Candida parapsilopsis, C. glabrata, C. albicans, C. neoformans, Trichophyton rubrum,* and *Microsporum gypseum* with values of MIC in the range of 4 to 64 µg mL^{-1} (Yu et al 2014). The sponges-associated fungi have also been contributed to be source of marine natural products. Irciniidae-associated microbes have successfully considered as a potential source of pharmaceutical agents (Hardoim & Costa 2014). For instance, sorbicillactone A produced by *Penicillium chrysogenum* was isolated from *Ircinia fasciculata,* exhibited cytotoxic effect against murine leukaemia lymphoblast (L5178y) cells and anti-HIV activity (Bringmann et al 2003). The other sponges-associated fungi, *Aspergillus flavus* GU815344 isolated from *Callyspongia* spp., has antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 29213 (Meenupriya & Thangaraj 2010). In addition, the sponges-associated fungi from Indonesia, *Trichoderma reesei* MG547722.1, was isolated from *Cynachirella* sp. that has antibacterial activity toward *S. enterica* ser. *thypii*, ESBL *E. coli,* and *S. haemolyticus* (Sibero et al 2018a). Nevertheless, the exploration of sponges-associated fungi that have anti *A. baumannii* activity is rare. Therefore, the sample sponges obtained from Karimunjawa National Park would be a great source to acquire potential fungi with antibacterial activity against MDR *A. baumannii.*

Isolation and purification of sponges-associated fungi. Isolation of fungi associated from 13 sponges resulted 24 fungi with various morphology. The isolates consisted of

molds such as *Aspergillus, Fusarium, Trichoderma,* and *Penicillium*; and some yeasts. The fungal diversity is shown by Figure 3. The similar method was conducted by Sibero et al (2017b) to isolation sponges-associated fungi from Teluk Awur and Panjang Island, Jepara, Indonesia. Isolation method and medium have an important role to the diversity of sponge-associated fungi (Ding et al 2011; Nguyen et al 2018; Overy et al 2019).

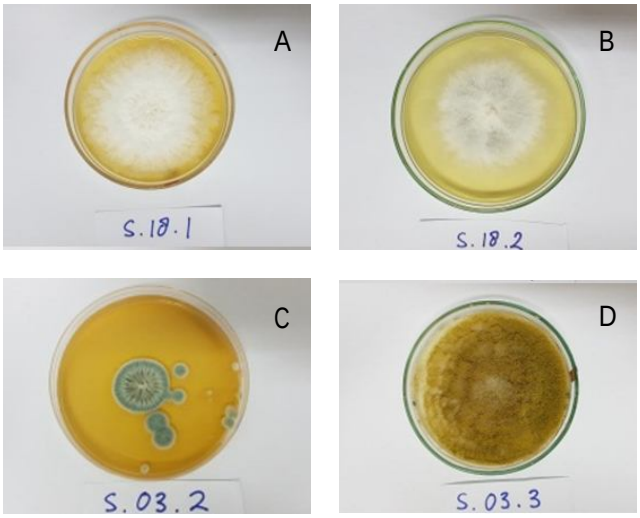

Figure 3. Some sponges-associated fungi were isolated from Karimunjawa National Park (A-B: *Fusarium* sp., C: *Penicillium* sp., D: *Aspergillus* sp.).

Multidrug resistant test. In this study, the susceptibility of *A. baumannii* on several antibiotics was tested to understand the resistance status of the clinical isolate. The susceptibility of this bacterium to antibiotic was showed in Table 1. According to the result, the clinical isolates of *A. baumannii* were resistant to eleven antibiotics. These antibiotics represented seven classes of commercial antibiotics. There were Penicillin (β-lactam), Penicillin (β-lactamase inhibitor), Cephalosporin, Monobactam, Carbapenem, Fluoroquinolone, and Sulfonamides.

Table 1

Susceptibility information of *A. baumannii* for multidrug resistant test. The interpretation of R is resistant to antibiotic while S is susceptible and I is intermediete

Antibiotics	*A. baumannii*	
	MIC	*Interpretation*
Ampicillin	≥ 32	R
Ampicillin / Sulbactam	≥ 32	R
Piperacillin / Tazobactam	≥ 128	R
Cefazolin	≥ 64	R
Ceftazidime	≥ 64	R
Ceftriaxone	≥ 64	R
Cefepime	≥ 64	R
Aztreonam	≥ 64	R
Meropenem	≥ 16	R
Amikacin	4	S
Gentamicin	8	I
Ciprofloxacin	≥ 4	R
Tigecycline	2	S
Nitrofurantoin	≥ 512	R
Trimethoprim / Sulfamethoxazole	≤ 20	S

Table 1 presents the results of susceptibility test of the clinical *A. baumannii* that was used in this study. This pathogen was resistant to 11 antibiotics that represented sevenclasses of antibiotic. There were Penicillin (beta-lactam), Penicillin (beta-lactam)

and beta lactamase inhibitor, chepalosporin, monobactam, carbapenem, fluoroquinolone, and sulfonamides. According to Magiorakos et al (2012), bacteria which resistant to three or more antibiotic classes could be highlighted as multidrug-resistant organism. Due to the result of susceptibility test, we confirmed the clinical *A. baumannii* in this study was MDR.

Preliminary antibacterial test. Agar plug method was conducted as a preliminary screening of antibacterial activity from sponge-associated fungi to obtain the prominent isolate for the further step. Six of all the isolates successfully inhibited MDR *A. baumannii* including KJ-S16.1, KJ-S03.1, KJ-S26, KJ-S30.1, KJ-S30.2 and KJ-N25 (Table 2). The active isolates were found from sponges that collected from sites 1, 3, and 4. The isolate KJ-S03.1 showed the most potential activity against MDR *A. baumanni* from the diameter of clear zone. Balouiri et al (2016) stated that during growth on agar, microorganisms secret particular metabolites into the media. In agar plug method, the fungi on its agar media which contain secreted metabolites is placed onto another agar media which has been inoculated with pathogen. The metabolite in the fungal media will be transferred into the pathogen media, then kill or inhibit the growth of the pathogen that is confirmed by the presence of clear zone (Sibero et al 2018c).

Table 2

Preliminary antibacterial test from sponge-associated fungi against MDR- *A. baumannii*

Isolate	*Antibacterial activity*
KJ-S16.1	+
KJ-S16.2	-
KJ-S16.3	-
KJ-S18.1	-
KJ-S18.2	-
KJ- S03.1	+
KJ-S03.2	-
KJ-S03.3	-
KJ-S03.4	-
KJ-T17.1	-
KJ-T17.2	-
KJ-T17.3	-
KJ-N16	-
KJ-N21	-
KJ-N23	-
KJ-S26	+
KJ-S27.1	-
KJ-S27.2	-
KJ-S30.1	+
KJ-S30.2	+
KJ-S102.1	-
KJ-S102.2	-
KJ-N25	+
KJ-S47	-

Secondary metabolite extraction from the potential isolate. The purpose of secondary metabolite extraction from the potential isolate was to obtain the extracellular bioactive compound from the broth medium, during the 14 days of cultivation. The yield of 100 mL in the broth culture (marine MEB) was obtained a total 30.8 mg crude extract with pasta appearance in red color. Secondary metabolites were produced intracellular and extracellular. However, the antibacterial activity of intracellular was very weak (Sabdaningsih et al 2017; Sibero et al 2019b). Therefore, this study only utilize the extracellular metabolites.

Antibacterial susceptibility test. The crude extract of fungus KJ-S03.1 was tested against MDR *A. baumannii* with concentration 100 µg mL^{-1}. A positive control was used to

ensure that antibacterial activity present in the crude extract was detected. Chloramphenicol 50 µg was used as a positive control, while the negative control in this study used organic solvent (EtOAc). The average diameter of inhibition zone was 4.95±0.35 mm which is shown in Figure 4. Sibero et al 2017a also found the anti-MDR *A. baumannii* activity with diameter of inhibition zone at 4.25 mm ± 0.35 from fungal red pigment RS 1A isolated from ant plant (*Hydophytum formicarum*). The low antibacterial activity of this fungi, may be affected by the ability of *A. baumannii* to produce biofilm (Peleg et al 2008). Biofilm contains exopolysaccharide which make the bacteria be thicker and having more surviving capabilities in stress conditions. Therefore, *A. baumannii* could defend from the activity of KJ-S03.1 crude extract.

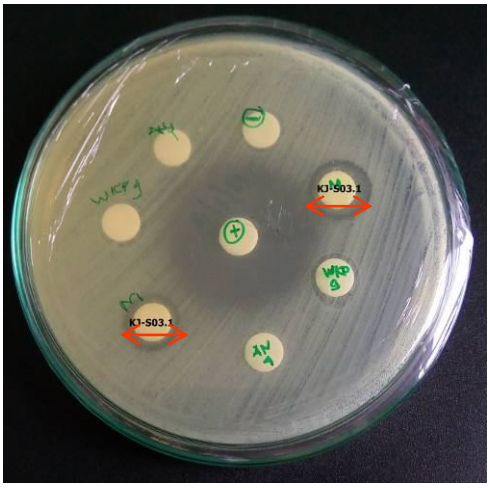

Figure 4. Inhibition zone of the KJ-S03.1 crude extract against MDR *A. baumannii* was showed by red arrows.

Sponge identification of the potential isolate. The identification of sponge was conducted using microscopic technique. The observation of ectosome and choanosome was showed in Figure 5, while the spicules were presented in Figure 6.

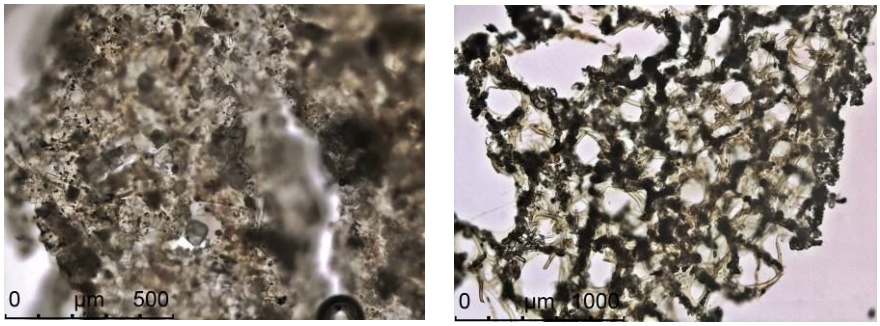

Figure 5. Section tissue of sponge KJ-S.03 (ectosome: left; choanosome: right).

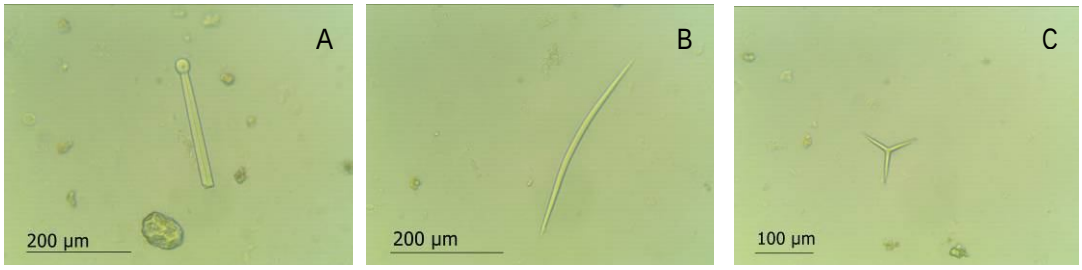

Figure 6. Light microscope images from spicules of sponge KJ-S.03 under microscope observation with magnification 40 x 10 (A: monaxone, B: diaxone, C: triaxone).

According to the observation under microscope, this sponge KJ-S.03 has found three different megascleres. There were monaxone (Figure 6A), diaxone (Figure 6B), and

triaxone (Figure 6C) expected as *Carteriospongia* sp. This sponge has plate-like (lamellate) shape with the ridged surface in green color. The appearance texture is sandy, due to incorporated sand and debris in the ectosome with the size about 50 cm (Goudie 2011). The distribution is strictly found in Indo-West Pacific. The megascleres spicule skeleton strength the structure of sponge (van Soest et al 2012).

Molecular identification of the potential isolate. To understand the species of prospective isolate, molecular identification was conducted. Table 3 shows the result of homology analysis from a sequence of KJ-S03.1 and Figure 7 shows a phylogenetic tree of this potential isolate. According to molecular identification in ITS region, fungus KJ-S03.1 had 99% similarity to *Aspergillus nomius* (593 bp, KR296847.1). Genus *Aspergillus* has been noted as a cosmopolite microorganism because it could be isolated from various sources. Rani et al (2017) successfully isolated *A. nomius* from medicinal plant *Calotropis procera* with antibacterial activity against *Salmonella typhi*. *A. nomius* was also isolated from bees (Massi et al 2015), Japanese-tea field soil and silkworm excrements (Ito et al 1998), local landfill soil (Munir et al 2018), spices (Garcia et al 2018), dairy cattle feed (Variane et al 2018) and cocoa (Copetti et al 2011). Therefore, KJ-S03.1 was considered as marine-derived fungi (Overy et al 2019). The partial sequences of 18S rRNA gene of this fungi was deposited into Genbank with accession number LC415575.1.

Table 3

Homology analysis of the sequence of KJ-S03.1 based on BLAST

Isolate code	*Close relative*	*Homology*	*Acc. number NCBI*
KJ-S03.1	*Aspergillus nomius*	99%	KR296847.1

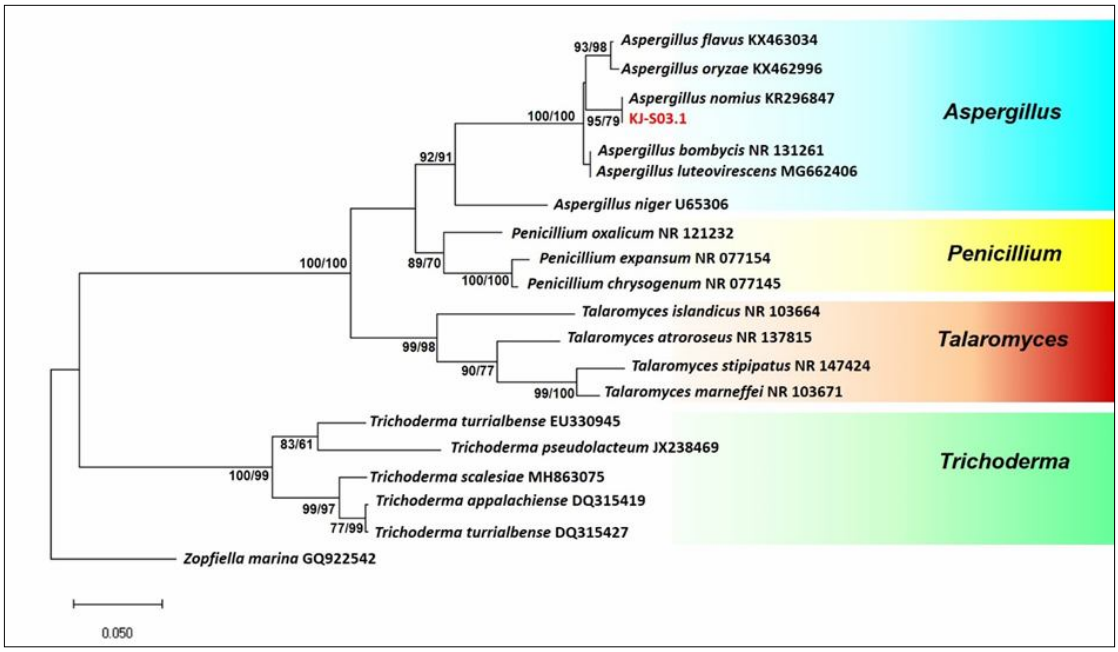

Figure 7. A Maximum likelihood phylogenetic tree based on Internal Transcribed Spacer (ITS) region, with 1000 bootstrap replications. The number of each node presents bootstrap values from Neighbor-Joining (NJ) and Maximum likelihood (ML). Sponge-associated fungi is indicated by red letters.

Metabolite characterization. The observation of substances in KJ-S.03.1 was conducted using TLC. The objective of this step was to characterize the bioactive compounds in fungal extract. Separation of KJ-S.03.1 extract in dichloromethane: ethyl acetate: methanol (6:4:1, v/v) was showed in Figure 8. There were 14 spots based on UV absorbtion. Most of them were found as semipolar and nonpolar compounds.

Phytochemical test has obtained the group of substances which contained in the crude extract from KJ-S03.1 (Table 4). The crude extract involved to alkaloid, quinone, and flavonoid. Kristanti & Tunjung (2013) also detected alkaloid in Rf score 0.8 and flavonoid 0.9. Alkaloids are one of the natural products which have a diverse group of compounds with antibacterial activity. For instance, squalamine, a polyamine alkaloid isolated from the dogfish shark has potential to inhibit Gram-negative pathogens more susceptible than ciprofloxacin (Cushnie et al 2014). In the other hand, the antibacterial activity of quinone has been studied since 1943 by Armstrong and colleagues (Armstrong et al 1943). Flavonoid is also well-known as an antibacterial agent, the hydroxyl group attached to the aromatic rings is important to increase the antibacterial activity (Xie et al 2015).

Figure 8. LC Si-60 F_{356} plates of crude extracts of KJ-.03-1.

Table 4

Phytochemical test from KJ-S03.1

Parameter	*Test result*
Alkaloid	+
Quinone	+
Tannin	-
Saponin	-
Flavonoid	+

Conclusions. The study has revealed a total 24 sponges-associated fungi isolated from thirteen sponges where taken at Karimunjawa National Park. There were six potential isolates which active against MDR *A. baumannii.* This strain has resistance to 11 commercial antibiotics. The potential fungi was encoded by KJ-S.03.1, according to ITS region and partial 18S rRNA sequence, this isolate was identified as *Aspergillus nomius.* The crude extract was performed using TLC, obtained 14 spots and contained alkaloid, quinone, flavonoid, as well. Isolate *A. nomius* KJ-S03.1 is a potential source of antibacterial agent that active against the MDR *A. baumannii.*

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Authors:
Aninditia Sabdaningsih, Department of Aquatic Resources Management, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: aninditiasabdaningsih@live.undip.ac.id
Olvi Cristianawati, Doctoral Program of Coastal Resources Management, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: olvi.cristiana@gmail.com
Mada Triandala Sibero, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: madatriandala@hotmail.com
Muslihuddin Aini, Marine Natural Product Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: muslihuddin.aini@gmail.com
Ocky Karna Radjasa, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: ocky_radjasa@yahoo.com
Agus Sabdono, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: agus_sabdono@yahoo.com
Agus Trianto, Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Diponegoro University, 50275 Tembalang, Semarang, Central Java, Indonesia, e-mail: agustrianto.undip@gmail.com
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