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Agelasines B, D and antimicrobial extract of a marine sponge *Agelas* sp. from Tahuna Bay, Sangihe Islands, Indonesia

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Abstract. *Balansa W, Wodi SIM, Rieuwpassa FJ, Ijong FG. 2020. Agelasines B, D and antimicrobial extract of* Agelas *sp. from Tahuna Bay, Sangihe Islands, Indonesia. Biodiversitas 21: 699-706.* The alarming growth of antibiotic-resistant bacteria necessitates the discovery of new antibiotics including those for combating life-threatening ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp) and fish pathogenic bacteria. This study aimed to identify antimicrobial compounds from an extract of a marine sponge collected from Enepahembang coral reef, Sangihe Islands, North Sulawesi, Indonesia. The sponge was identified by DNA barcoding as *Agelas* sp. and its extract was evaluated against three ESKAPE bacteria (*S. aureus*, *K. pneumoniae*, and *A. baumannii*) and three fish pathogenic bacteria (*A. hydrophila*, *Edwardsiella tarda* and *Vibrio parahaemolyticus*), using the standard disk diffusion method. It showed moderate to strong antimicrobial activity against *S. aureus* (25.3 mm), *K. pneumoniae* (15.5 mm), *A. baumannii* (20.2 mm), *A. hydrophila* (20.5 mm), *E. tarda* (22.4 mm) and *A. salmonicida* (21.2 mm). The extract was isolated by chromatographic techniques (column chromatography, flash chromatography, and high-performance liquid chromatography). The structures and relative stereochemistry of the two compounds were elucidated by HRESIMS, 1D and 2D NMR data analysis as well as by comparison with reported values. Unfortunately, limited amount of the pure compounds prevented us from further evaluating their antimicrobial activity against the test bacteria. Nevertheless, the crude extract's strong antimicrobial activity, especially against the test Gram-negative bacteria, suggests the importance of this finding in light of the recent antimicrobial drug scarcity but rapid antimicrobial resistance and the emerging paradigm of antimicrobial drug modification, redirection and/or repurposing for discovering new antibiotics particularly against the life-threatening Gram-negative bacteria.

Keywords: *Aeromonas hydrophila*, *Agelas* sp., *Edwardsiella tarda*, Enepahembang, ESKAPE bacteria, Sangihe Islands

Abbreviations: ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* sp.

INTRODUCTION

The ESKAPE bacteria are the major cause of life-threatening nosocomial infections and are highly efficient in becoming resistant to various antimicrobial agents (Navidinia 2016). Because of their tremendous clinic and economic impacts, contributing to high mortality and health care costs (Founou et al. 2017), the World Health Organization (WHO) has listed the ESKAPE pathogens in the 12 bacteria with the most urgent need of antibiotics (Tacconelli et al. 2018). Of these, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter* spp and *Enterococcus faecium* (VRE) were classified as critical priority and *S. aureus* (MRSA and VRSA) as high priority group (WHO 2017). Similarly, there is an alarming increase in resistance of fish pathogens such as *Aeromonas hydrophila*, *Edwardsiella tarda* and *Aeromonas salmonicida* against the commercial antibiotics, which were recently reported to be partially effective against the fish pathogens (Miller and Harbottle 2018). Moreover, the ability of fish pathogens such as *Aeromonas* spp., *Edwardsiella* spp. and *Vibrio* spp. to transfer antibiotic-resistant genes to other fish pathogens exacerbates the current antibiotic resistance crisis (Miller and Harbottle 2018). Together, these facts strongly suggest a desperate need for new antibiotics to combat these deadly infectious bacteria (Tommasi et al. 2015).

In our ongoing investigation of new antibiotic drug leads from sponges of Sangihe Islands, North Sulawesi Indonesia (Balansa et al. 2017, 2019; Azhari et al. 2018), we noticed that the crude extract of a sponge collected in Enepahembang East Tahuna showed a strong antibacterial activity against three ESKAPE bacteria (*S. aureus*, *A. baumannii* and *K. pneumoniae*) and three fish pathogenic bacteria (*A. hydrophila*, *V. parahaemolyticus*, and *E. tarda*). This encouraged us to further identify the sponge species and investigate its active component, leading to the discovery of agelasines B and D from an *Agelas* sp. Over the years, agelasines have been reported to exhibit a broad range of bioactivities including antifungal against *Cryptococcus neoformans*, antileishmanial against *Leishmania donovani* and antibacterial against *S. aureus* and methicillin *S. aureus* (Yang et al. 2012), antiprotozoal against *Leishmania infantum* and *Trypanosoma cruzi* (Vik

et al. 2009), antimalarial against *Plasmodium falciparum* (Appenzeller et al. 2008), antifungal against *Candida albicans*, antimicrobials against *Staphylococcus aureus, Bacillus subtilis* (Capon and Faulkner 1984), *Micrococcus luteus* (Vik et al. 2006), antimycobacterial against both dormant and active mycobacterium (Arai et al. 2014) and an inhibitor of Na^+ and K^+ ATPase (Nakamura et al. 1984). In particular, both agelasines B and D were reported to have anticancer against PC9, A549, HepG2, MCF-7, and U937 cancer cell lines (Hong et al., 2017) and a potent antimicrobial activity against *S. aureus* (Vik et al. 2006). To date, however, almost nothing is known about antimicrobial potential of agelasines against both Gram-negative ESKAPE (*A. baumanii* and *K. pneumoniae*) and fish pathogenic (*V. parahaemoliticus*, *E. tarda* and *V. salmonicida*) bacteria. This article reports structure elucidation of agelasines B and D and antibacterial of the crude extract of *Agelas* sp. towards fish pathogenic and ESKAPE bacteria.

MATERIALS AND METHODS

Sample collection

With the help of self-contained underwater breathing apparatus (SCUBA), the specimen was collected on July 5, 2017 by hand from Enepahembang, East Tahuna, Sangihe Islands in North Sulawesi Province, Indonesia at a depth of ~ 6 m and geographical position of 3°36′00.7″N, 125°29′44.5″E (Figure 1). Soon after morphological description and underwater photograph, the specimen was cut (<10% of the total mass), individually kept in a zipped plastic bag and brought to the laboratory in Nusa Utara Polytechnic, Tahuna, North Sulawesi, Indonesia where the specimen was stored at a -16°C freezer until used.

Test bacteria

The isolates were obtained from the American Type Culture Collection including *Aeromonas hydrophila* (ATCC 35654) *Vibrio parahaemolyticus*, *Edwarsiella tarda* (ATCC 33658), *Staphylococcus aureus* sub. (ATCC 25923), *Acinetobacter baumannii* (ATCC 19606), and *Klebsiella pneumoniae* (ATTC BAA-1705).

Antibacterial evaluation

Nutrient agar and 1% marine broth were prepared for *Acinetobacter baumannii* and *Klebsiella pneumoniae* and nutrient agar + 1% Brain Heart Infusion (BHI) Broth was prepared for *Staphylococcus aureus*. The bacteria were streak on media and were left for 2 hours before antibacterial test. For the test, disc diffusion method was adopted. Blank disk Whatman papers (6 mm) were impregnated with 20 µL of extract and/or antibiotic, positioned on the agar plate after dry and allowed to dry in an incubator at 37°C for 12-24 hours before the measurement of inhibition zone around the paper disk.

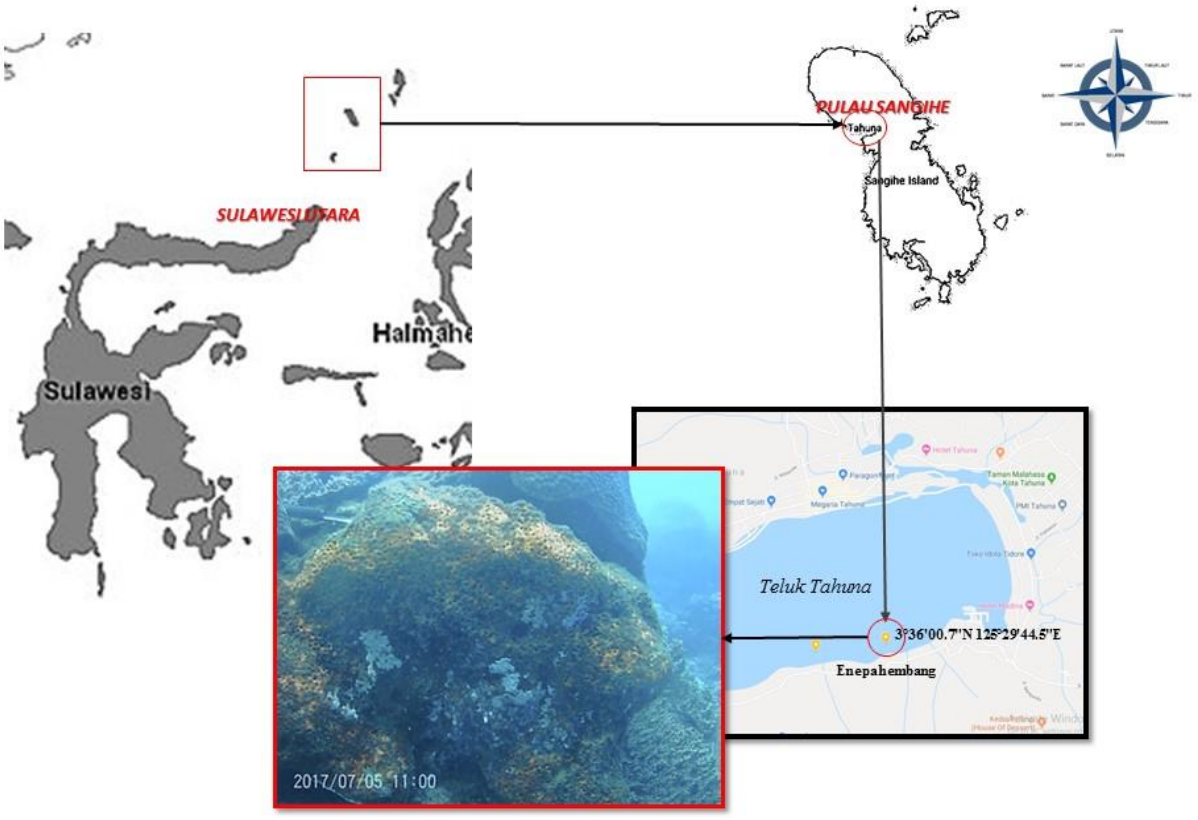

Figure 1. Map of sampling location in Enepahembang Sangihe Islands, North Sulawesi, Indonesia and the specimen with the image in the lower right-hand corner showing the location site (accessed from Google map in October 2019) and the front image showing underwater photograph of the sponge *Agelas* sp.

Table 1. Comparison of NMR data of agelasine B (DMSO) and Compound **1** (CD_3OD)

#	δ_H (mult., *J* (Hz)) (+)-Agelasine B (DMSO)		δ_H (mult., *J* (Hz)) comp. 1 (CD_3OD)	
1	1.54, m	17.8, CH_2	1.53, m	18.2, CH_2
2	1.98, m	26.3, CH_2	2.04, m	29.1, CH_2
3	5.13, s	120.2, CH	5.21, s	120.3, CH
4		143.6, C		145.1, C
5		37.6, C		38.5, C
6a	1.65, dt (13.2, 3.0)	36.2, CH_2	1.60 m	36.3, CH_2
6b	1.10, m			
7a	1.46, m	27.0, CH_2	2.01 m	28.6, CH_2
7b	1.38, m		1.48 m	
8	1.42, m	35.7, CH	1.50 m	36.1, CH
9		38.2, C		38.2, C
10	1.29	45.9, CH	1.50 m	48.1, CH
11a	1.47	35.8, CH_2	1.60 m	36.2, CH_2
11b	1.35			
12a	1.97	32.5, CH_2	2.15 m	34.1 CH_2
12b	1.84		2.05 m	
13		146.2, C		150.0, C
14	5.45, t (6.0)	114.9, CH	5.57, t (6.0)	115.4, CH
15	5.16, d (6.0)	47.0, CH_2	5.19 br s	47.9, CH_2
16	1.79, s	16.7, CH_3	1.75, s	16.7, CH_3
17	0.78, s	15.8, CH_3	0.87, d (1.8)	14.5, CH_3
18	1.54, d (1.2)	17.7, CH_3	1.60 (s)	18.7, CH_3
19	0.95, s	19.6, CH_3	1.06 (s)	20.1, CH_3
20	0.70, s	18.1, CH_3	0.79 (s)	18.3, CH_3
2′	8.48, s	155.4,CH	8.48 (s)	157.1, CH
4′		148.9, C		150.1, C
5′		109.2, C		115.2, C
6′		152.3, C		152.0, C
8′		140.9, CH		142.0, C
9′-CH_3	3.89, s	31.4, CH_3	4.00 (s)	31.1, CH_3

Sample preparation

The sponge (500 g wet weight) was cut into small pieces and dried in the oven at 45^0C for two days before used. The dried specimen (49.3 g dried weight) was soaked in 400 mL MeOH overnight (2x), filtered and the combined supernatants were dried under a reduced pressure to yield 4.1g crude extract. It was triturated with hexane, dichloromethane, methanol, and H_2O. The MeOH soluble was further subjected to separation with SiO2 gel column chromatography (gradient elution 90% Hexane to 100% DCM followed by 90% EtOH to 100% MeOH to give 10 fractions, two of which (fractions 6 and 7) were further fractionated by normal phase column chromatography (column F0220-205 g, 20 bar, gradient elution 0-100% (Hexane, DCM, EtOAc) to 10% MeOH, flow rate 127 mL/min for 40 m) to afford 13 fractions.

Of these, the active sub-fraction 10 (141 mg) was further subjected to a repeated reversed-phase flash chromatography (the Interchim Puriflash 4125 chromatography system applied with a Puriflash C_{18}-HP30 mm Flash column, gradient elution of 5% MeOH/H_2O to 100% MeOH over 1 h) to give 10 fractions. Fraction 5 (25.0 mg) was further purified by HPLC (semi preparative column, EC Gravity C_{18}, 250 x 10 mm, gradient elution, 88% MeOH/H_2O to 95% MeOH/H_2O + 0.01% TFA, flow rate 3.0 mL/min., over 30 min.) to give 10 subfractions. Further purification of fraction 2 by HPLC (semi preparative column, EC Gravity C_{18}, 250 x 10 mm, gradient elution, 88% MeOH/H_2O to 95% MeOH/H_2O + 0.01% TFA, flow rate 3.0 mL/min., over 45 min.) resulted in the isolation of agelasine B (1) (1.05 mg) and agelasine D (2) (0.9 mg).

Spectroscopic analysis

NMR spectra were recorded in MeOH-d_4 (ALDRICH, St. Louis, MO, USA) or chloroform-d with tetramethylsilane as an internal standard (ALDRICH, St. Louis, MO, USA), using a Bruker AV400 and 600 MHz NMR spectrometer for 1D and 2D NMR data respectively (Brucker, Ettlingen, Germany). Mass spectra were recorded on a micrOTOF-Q mass spectrometer (Bruker, Billerica, MA, USA) with ESI-source coupled with a HPLC Dionex Ultimate 3000 (Thermo Scientific, Darmstadt, Germany) using an EC10/2 Nucleoshell C18 2.7 µm column (Macherey-Nagel, Düren, Germany). The column temperature was 25°C. MS data were acquired over a range from 100 to 1000 *m/z* in positive mode. Auto MS/MS fragmentation was achieved with rising collision energy (35-50 keV over a gradient from 500 to 2000 *m/z*) with a frequency of 4 Hz for all of the ions over a threshold of 100. The injection volume was 2 µl with a concentration of 1 mg/mL. Fractionation was performed on the Interchim Puriflash 4125 chromatography system and purification on a Shimadzu HPLC equipped with UV Vis and diode array.

Identification of the sponge

A small piece of the sponge specimen was added into 400 µL lysis mix (100 mM NaCl, 50 mM 191 Tris-HCl pH 8.0, 10 µM EDTA pH 8.0, 0.5% SDS, 2 mg/mL Proteinase K) and incubated at 56°C overnight (Vargas et al. 2012). Thereafter, genomic DNA was isolated using innuPREP Bacteria DNA Kit (Analytik Jena, Jena, Germany) and dialyzed using a 0.025 µm nitrocellulose membrane (MerckTM MF-MilliporeTM, Ireland). Prior to PCR, the sponge genomic DNA was diluted 1:10. The following barcoding primers were used (Chombard et al. 1998): C2: 5′-GAAAAGAACTTTGRARAGAGAGT- 3′ and D2: 5′-TCCGTGTTTCAAGACGGG-3′ to amplify the 28S rRNA fragment from the isolated sponge DNA. PCR program: initial denaturation at 95°C for 5 minutes; 34 cycles of 95°C for 45 seconds, 48°C for 45 seconds, 72°C for 30 sec and final elongation at 72°C for 5 min. In addition to that, a fragment of the mitochondrial cytochrome oxidase subunit 1 (CO1) was amplified using degenerated CO1 barcoding primers: dgLCO1490: 5′-GGT CAA CAA ATC ATA AAG AYA TYG G-3’ and dgHCO2198: 5′-TAA ACT TCA GGG TGA CCA AAR AAY CA-3’. PCR program: initial denaturation at 95°C for 2 minutes; 37 cycles of 94°C for 40 seconds, 42°C for 40 seconds, 72°C for 1 minute and final elongation at 72°C for 5 min. The amplified 28S rRNA and CO1 fragments were sent to Microsynth Seqlab,

Göttingen, Germany, for Sanger sequencing. Sequence reads were assembled in Clone Manager 9 and aligned by BLAST (NCBI) to determine the sponge identity.

RESULTS AND DISCUSSION

Collection site and the sponge

The orange, massive, rounded and encrusting sponge was consistently firmly spongy, resilient when wet and hardly compressible when dry. The exterior was reddish-brown to brick red and the interior had similar color but somewhat lighter when dry. The description matched the genus *Agelas* (Hoshino 1985) and was further supported by DNA barcoding analysis, allowing the assignment of the sponge as *Agelas* sp.

Extraction and purification of Agelasines B and D

The marine sponge (500 g, dry weight) was extracted with MeOH (2x 500 mL). The residue was partitioned between Hexane, DCM, MeOH, and H_2O. One of the active fractions (MeOH soluble) was passed through a chromatography column of silica gel 60 and eluted with hexane, DCM, EtOAc, and MeOH to obtain 13 fractions. Fraction 8 of this column was subjected to repeated separation with both normal and reversed-phase flash chromatography and followed by repeated purification by a reversed-phase high-performance liquid chromatography (HPLC) to give agelasine B (**1**) and agelasine D (**2**). The molecular formula of $C_{26}H_{40}N_5$ for both compounds was deduced from the HRESIMS. The ^{1}H NMR of **1** revealed the presence of two olefinic protons at δ_H 5.21 (H-3) and δ_H 5.57 (H-14), one doublet methyl at 0.87 δ_H (H-17), two singlet methyls at δ_H 1.06 (H-19) and at δ_H 0.79 (H-20), one vinylic methyl at δ_H 0.83 (H-18), four methylene protons at δ_H 1.53 (H-1), δ_H 2.04 (H-2), δ_H 1.60 (H-6) & δ_H 2.01 (H-7) and one methine proton at δ_H 1.50 (H-8), indicative of a clerodane moiety in **1**, which was further supported by the ^{13}C and HSQC data for C1 to C20 (Table 1). In addition, the 1D NMR data of 1 showed one olefinic proton at δ_H 8.48 (H-2′), one vinylic proton at δ_H 1.75 (CH_3-16) and one *N*-methyl at 4.0 (CH_3-9′), three quaternary carbons at δ_C 150.1 (C-4′), 115.2 (C-5′) and 152.0 (C-6′), two sp^2 carbons at δ_C 157.1 (CH-2′) and 142.0 (CH-8′) as well as one *N*-methyl carbon at δ_C 31.1 (CH_3-9′), suggestive of a purine moiety in **1** (blue, Figure 2 (A). *E*-configuration at C-13 was assigned based on the upfield chemical shift of CH_3-16 (δ_C 16.7) (De Rosa et al. 1976). Thus, **1** is a terphenyl purine derivative, featuring a bicyclic diterpene connected to position 7 of a 9-methyladenium chromophore, similar to that of agelasine A or B (Gordaliza 2010). However, the bridgehead methyl at CH_3-19 of **1** had a high field resonance at δ_C 19.5, suggesting a *trans*fused clerodane stereochemistry for **1** (pink, Figure 2 (A), matching that of agelasine B (Nakamura et al. 1984). This was further supported by COSY and HMBC correlations, establishing connections for clerodane diterpene, *N*-methyladenium and the two functionalities (Figure 2, (A)- (C)). Therefore, compound **1** was assigned as agelasine B.

Comparison of the ^{1}H NMR spectra between agelasines B (**1**) and D (**2**) showed a striking similarity (Figure 3). The only difference lied in the disappearance of a vinylic proton at δ_H 5.21 (H-3) and a methine proton at δ_H 1.50 (H-8) from the ^{1}H NMR spectrum of **2** in exchange of two exo-olefins at δ_H 4.56 (H-17a) and at δ_H 4.59 (H-17b), methine proton at 1.52 (H-9) and methine proton and the shift of a bridgehead proton from δ_H 1.06 (H-19) δ_H to δ_H 0.97 (H-20) in **2**, indicative of a rearranged labdane in **2** as shown. The ^{1}H NMR data of **2** matched those of agelasine D (Nakamura et al. 1984) and ageloxime D (Yang et al. 2012). In particular, the comparison of the ^{1}H NMR spectrum of **2** with that of ageloxime showed an exact match (Yang et al. 2012 and its supporting information). Therefore, compound **2** was tentatively assigned as having similar stereochemistry to agelasine D (Nakamura et al. 1984) and ageloxine D (Yang et al. 2012) but the same structure with that of agelasine **D** (Nakamura et al. 1984).

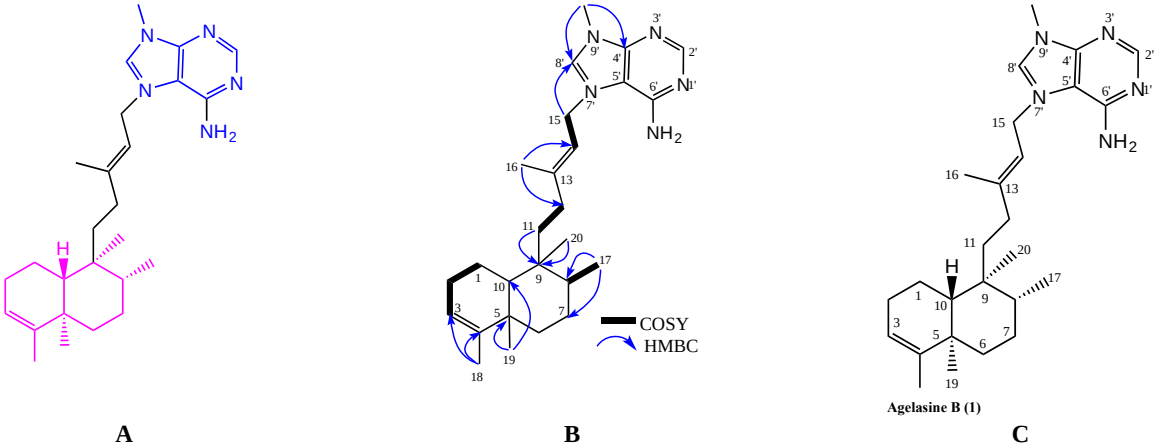

Figure 2. Clerodane and methyladenium functionalities (A), selected COSY and HMBC correlations (B) and agelasine B (**1**) (C)

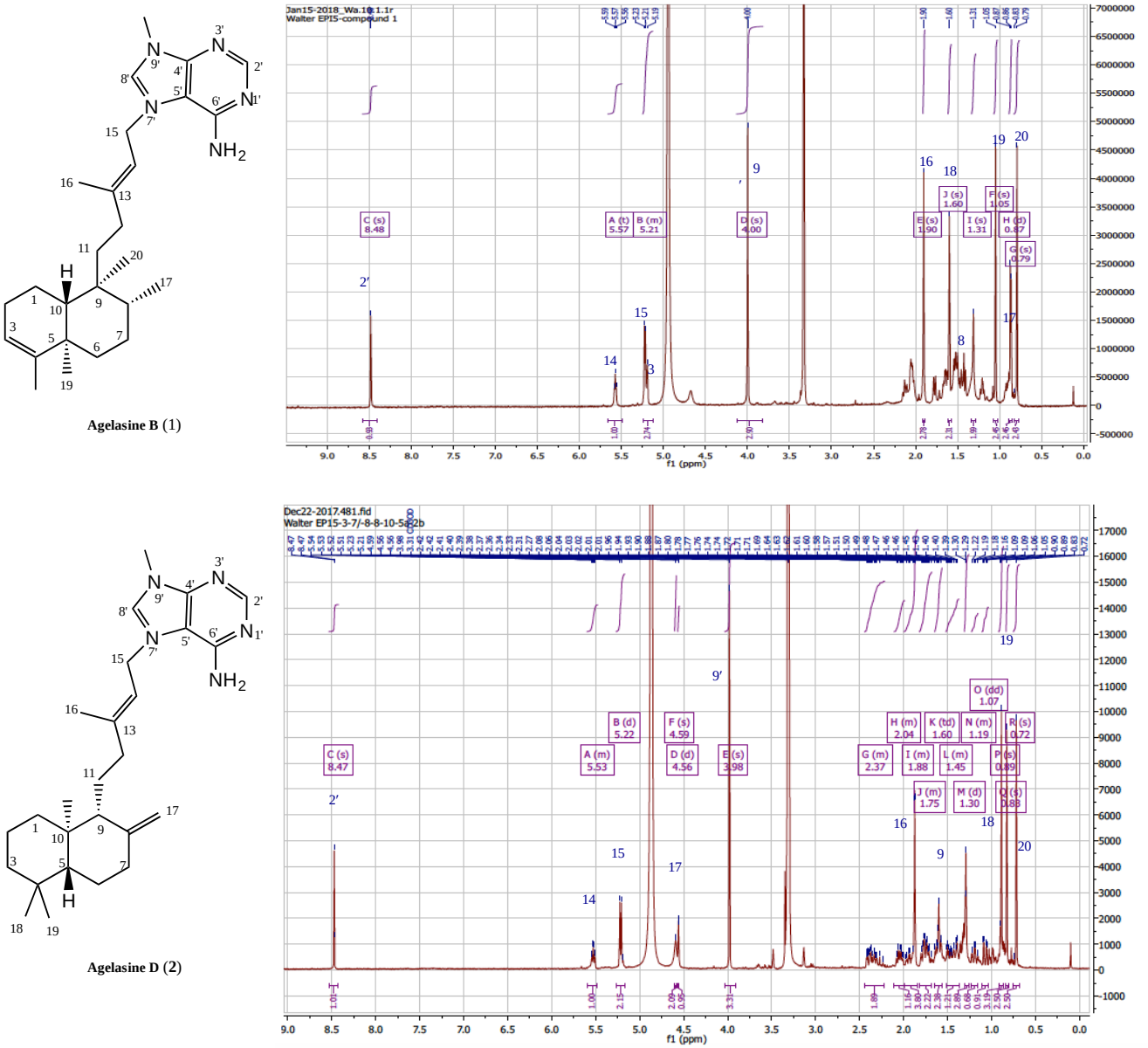

Figure 3. The ^{1}H NMR spectra of agelasine B (**1**) (*above*) and agelasine D (**2**) (*below*), showing the key protons for the two compounds

Antimicrobial screening

The standard disk diffusion method was used to screen antibacterial activity especially for the crude extract of Sangihe *Agelas* sp. with inhibition zone of the extract against the test bacteria shown in Table 2.

The crude extract of *Agelas* sp. showed a broad-spectrum antimicrobial activity. It exerted the strongest activity against the Gram-positive bacteria *S. aureus* with an inhibition zone of 25.3 mm, strong activity against the Gram-negative *E. tarda*, *A. salmonicida*, *A. hydrophila* and *A. baumannii* with inhibition zones of (22.4, 21.2, 20.5 and 20.2) mm respectively and moderate activity against *K. pneumoniae* with an inhibition zone of 15.5 mm. Except for *K. pneumoniae*, the antimicrobial activity of the extract against all test bacteria was relatively weaker (20.2-25.3 mm inhibition zone) than that of the commercial antibiotics (tetracycline) (21.3-27.0) mm inhibition zone. Unfortunately, the scarcity of the compounds—due to repeated purification of agelasine type compounds and their isomeric nature—prevented us from confirming the activity of agelasines B and D against the test bacteria. Nevertheless, previous studies on antimicrobial activity of agelasines B and D showed that both compounds had potent antimicrobial activity against *S. aureus* (Vik et al. 2006; Hong et al. 2017). Also, agelasine D had a strong and broad-spectrum antimicrobial activity against *Mycobacterium tuberculosis* and both aerobic and anaerobic Gram-positive and negative bacteria (Hong et al. 2017). Taken together, they suggest that the antimicrobial activity of the Sangihe's *Agelas* sp. might be due to the presence of agelasines B and D.

Discussion

The result showed that Gram-positive bacteria were more sensitive (inhibition zone >25 mm) against the extract than the Gram-negative ones (inhibition zone <22.5 mm) at least in terms of inhibition zone. This agrees with the previous reports that claimed the sensitivity of the Gram-positive over the Gram-negative bacteria (Tadesse et al., 2008). When the researchers screened extracts of Norwegian sponges and ascidians for antibacterial activity, they discovered 29% of the extracts were active against Gram-positive bacteria but only 15% against Gram-negative bacteria. The same results were also found in various screening efforts on the Caribbean sponge with the Gram-positive being more sensitive towards extracts than their Gram-positive counterparts (McCaffrey and Andean 1985; Amade et al. 1987). This is even true for medical plant extracts. Koohsari et al. (2015), reported the susceptibility of Gram-positive bacteria over the Gram-negative bacteria with *S. aureus* being one the most susceptible Gram-positive bacteria while *Escherichia coli* and *Klebsiella pneumoniae* as the most resistant Gram-negative bacteria.

However, the antimicrobial activity of the extract against a range of Gram-negative bacteria is also encouraging. For one thing, the discovery of antibiotics against Gram-negative bacteria is always challenging. GlaxoSmithKline, for example, reported that the company did not discover any antibiotic hits even after screening 50,000 synthetic compounds against *E. coli* (Richter et al. 2017). Low permeability of Gram-negative bacteria's outer membrane is generally accepted as the major reason for the resistance of Gram-negative bacteria against many commercial antibiotics (Fair and Tor 2014). Hence, the ability of the *Agelas's* extract to exert antibacterial activity against various Gram-negative bacteria suggests that either agelasine B or D or the combination of these compounds have the capacity to penetrate the Gram-negative bacteria's outer membrane.

Table 2. Antibacterial activity of crude extract against various bacteria determined by the standard disk diffusion method..

Bacterial strains	Zone of inhibition (mm)			
	Metabolites of *Agelas* sp.			Standard antibiotic
	Crude extract (inhibition zone 15 uL, 10 mg/mL)	Agelasine B	Agelasine D	Tetracycline
Staphylococcus aureus ATCC 25923	25.3 mm	a	a	27.0 mm
Acinetobacter baumannii ATCC 19606	20.2 mm	a	a	21.3 mm
Aeromonas hydrophila ATCC 35654	20.5 mm	a	a	22.3 mm
Aeromonas salmonicida	21.2 mm	a	a	21.7 mm
Edwardsiella tarda ATCC 33658	22.4 mm	a	a	26.7 mm
Klebsiella pneumoniae ATCC BAA-1705	15.5 mm	a	a	14.0 mm

Note: a: No data obtained

Curiously, agelasines B and D contained a primary amine and a similar functional group, secondary amine, was reported to have a significant impact on antimicrobial activity of cholic acid derivatives (Li et al. 1999). Many other amine-containing compounds possessed bioactivity against Gram-negative bacteria. For another, it has been known that the most serious infection threat of Gram-negative bacteria might come from *Klebsiella pneumoniae* and *Acinetobacter* (Ventola 2015) and other Gram-negative ESKAPE bacteria due to their dual membrane envelopes that prevent many antibiotics from accessing their targets (Smith et al. 2018). This also means that the current result might be of interest for antibiotic drug lead discovery although such notion remains to be studied before any conclusion can be drawn.

Moreover, the result may also have relevance to the current emerging ideas of drug modification, redirection and repurposing (Richter et al. 2017; Canturri et al. 2019; Jang et al. 2019). Although antimicrobial drug repurposing is not a new concept, it is gaining momentum due to the alarming increase of antibiotic-resistant bacteria and the scarcity of available new antibiotics particularly for treating Gram-negative bacteria (Canturri et al. 2019). Through this approach, several drugs that were earlier not meant for antibiotics such as anthelmintic, anticancer, anti-inflammatory, immunomodulators, antipsychotic and antidepressant drugs have been repurposed, leading to the discovery of several new antibiotics including aurofonin, chlorocyclizine, and pentamidine (Fahra and Brown 2019). Over the years, agelasines are known for their broad range of bioactivities particularly as anticancer and antimicrobial agents with agelasine D proven to bind to BCG3185c protein as its main target in exerting its antimycobacterial activity against the Gram-negative bacteria *Mycobacterium tuberculosis* (Arai et al. 2014). Along with these facts, the present result indicates the possibility of agelasines B and/or D to be an antibiotic drug lead for treating Gram-negative bacteria.

Furthermore, the present result is presumably relevant for the current concept of drug redirection (Jang et al. 2019). With this approach, any known drugs (e.g. anticancer or other types of drugs) provided that they have antibacterial activity were redesigned or synthesized and later evaluated as antibiotics against the gram-negative bacteria (Jang et al. 2019). Early this year, Jang et al. (2019) reported that they obtained potent new antibiotic leads by slightly modifying the structure of the anticancer drug YM155 and its analogs. In fact, introduction of a methyl group on the purine of agelasine significantly improved the activity of agelasine type compounds against several Gram-positive bacteria, protozoans and cancer cell lines (Rogen et al. 2011). Modifications of the length, geometry, and purine moieties have also proven to improve the bioactivity of agelasine type compounds (Bakkestuen et al. 2005). To date, however, this effort is limited to targeting Gram-positive bacteria and *Mycobacterium tuberculosis* with the same effort on other Gram-negative bacteria is yet to be seen.

Another relevant concept with the present result is the design of molecules by focusing on the permeation and

accumulation of molecules into the Gram-negative bacteria's cells. Richter et al. (2017) reported that one of the keys for molecules to exert antibacterial activity against Gram-negative bacteria is to contain amine, have globular and rigid structure. The authors went on saying that although amine was not the only component needed for the efficacy of antibiotics against Gram-negative bacteria, this functionality permits molecules with this moiety to permeate through porin and accumulate in the bacteria cells, therefore exerting antibacterial activity against Gram-negative bacteria. Curiously, cholic acids containing three amine groups and cycloamide discovered through drug repurposing were reported as potent antibacterial that sensitized the Gram-negative bacteria (Richter et al. 2017). Thus, it is interesting to see whether or not the primary amine in purine functionality of agelasines B and D would play a key role in the current observed antimicrobial activity on various Gram-negative bacteria and whether or not the modification of this functionality will result in antibiotic drug leads.

The current result may also find relevancy in the idea of combining known compounds with one or two antibiotics to improve the activity of both new or the last resource antibiotics (Bassetti and Righi 2015). This approach has resulted in the discovery of promising antibiotic drug leads that are currently in phases 2 and 3 of the clinical trials (Bassetti and Righi 2015). A typical example is a combination of clavulanic acid and various β-lactam antibiotics, which restored the function of many β-lactam-containing antibiotics (Fahra and Brown 2019). Due to their broad range bioactivities and the present result, combination of agelasine B or D with commercial antibiotics would be an interesting alternative to discover antimicrobial drug leads for Gram-negative bacteria.

Therefore, this report provides evidence of antibacterial activity of extract of *Agelas* sp. from which agelasines B and D were isolated. In addition to providing isolation, purification and structure elucidation of agelasines B and D, this article describes the activity of the crude extract of *Agelas* sp. against a series of Gram-negative bacteria, which to our best knowledge represents the first report on the antimicrobial activity of agelasine type compounds against the Gram-negative fish pathogenic and ESKAPE bacteria.

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