Antibacterial potential of fungi derived extracts of marine sponge *Acanthostrongylophora ingens*

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Abstract. Sponge-derived fungi are potential sources of antibacterial agents which have an ability to produce a variety of unique and diverse bioactive metabolites. In our previous study, eight derived-fungi constituents were isolated from the marine sponge *Acanthostrongylophora ingens*. The aim of the present study was to test the antibacterial activity of ethyl acetate extract against *Vibrio cholerae* Inaba, *Enterococcus faecalis* ATCC 29252, Multidrug-resistant *Pseudomonas aeruginosa* (MDR-PA), *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* ATCC 25175, methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* ATCC 25923 and *Mycobacterium tuberculosis* H37Rv. One of isolated fungi IB141 showed significant capability to inhibit the growth of all above pathogenic bacteria except for *S. mutans* ATCC 25175. The antibacterial activity of ethyl acetate extract of fungus IB141 toward the above bacteria was tested by using agar diffusion method of Sabouraud Dextrose Agar (SDA) medium and showed significant activity against *S. aureus* ATCC 25923 and *M. tuberculosis* H37Rv using agar diffusion method of Lowenstein Jensen (LJ) medium to give strong inhibiton (no growth) at concentration of 0.025, 0.05 and 0.1%. This crude ethyl acetate extract was column chromagtographed on silica gel using an increasing amount of MeOH in CH_2Cl_2 and fractions obtained were checked by Thin Layer Chromatography (TLC), then the fractions having similar TLC pattern were combined to give four fractions. The second fraction (F2) was rechromatographed on silica gel eluted with CH_2Cl_2-MeOH (0.3:4.7) to give 7 subfractions. Subfractions F22, F23 and F26 were chromatographed on HPLC (reverse phase column, C_{18}) eluted with MeOH and the major peaks were compared to those of library hits of UV spectra data available in the Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University, Düsseldorf, Germany. It was concluded that the major component of subfraction F22 was palitantin (1) with retention time at 38.91 min, subfraction F23 citreodrimene A (2) with retention time at 36.98 min, and subfraction F26 septicine (3) and ochratoxin A (4) with retention time at 13.04 and 28.11 min, respectively.

Key Words: *Acanthostrongylophora ingens,* antibacterial activity, *Aspergillus ochraceus, M. tuberculosis* H37Rv.

Introduction. In recent decades the discovery of new antibacterial agents has proved to be very challenging for the research community (Boucher et al 2009; Nasr et al 2014). To solve this problem, one should look and find for new types of antibacterial agents. Therefore, most of the research focused on finding a new type or the design of new effective antibacterial agents (Fjell et al 2012; Giguère et al 2013).

One of antibiotic-producing sources was a fungus derived from a marine sponge. Marine derived-fungi have been repeatedly shown to have interesting sources of new secondary bioactive metabolites. Research on sponge-derived fungi has been widely reported and had variety of bioactivities such as antimicrobial, cytotoxic, anticancer, and antiinflammatory, among others (Lee et al 2013; Li et al 2013; Subramani et al 2013; Artasasta et al 2019; Sandrawati et al 2019). Recently, the authors conducted a preliminary study on the antibacterial and cytotoxic activity of derived-fungi of marine sponge *Acanthostrongylophora ingens* collected from Mandeh Island, West Sumatra. The fungi derived extract were found to be potential sources of antibacterial compounds as the extracts showed significant antibacterial activity against *Bacillus subtilis*,

Staphylococcus epidermidis, *Salmonella typhosa* and *Escherichia coli* (Handayani & Aminah 2017). Based on these findings it is interesting to continue and assess the activity of these fungi isolated constituents from this sponge against several pathogenic bacteria and resistant bacteria.

Material and Method

Sponge material. *A. ingens* specimens (Herbarium No. IB101) have been collected from the sea around Mandeh Island, West Sumatra in December 2015 and stored in the Marine Reference Collection, Sumatran Biota Laboratory, Andalas University, West Sumatra, Indonesia. Sponge *A. ingens* was collected from the Mandeh Island, south coast of West Sumatra, Indonesia, in depth of ±5-8 m. Sponge was transferred into a sterilized plastic bag and stored in the ice box. The samples were transferred to the laboratory and processed immediately for the isolation of symbiotic fungi. The sponges were identified by Dr. Nicole J. De Voogd, Center for Nature Biodiversity, Netherlands.

Isolation, cultivation, and extraction of secondary metabolites of derived fungi. Isolation and cultivation of derived fungi using rice medium and incubated at room temperature for 4-6 weeks until the volume of rice in the Erlenmeyer is overgrown by the fungi. This method has been carried out following the methods of Kjer et al (2010), and Handayani & Aminah (2017).

Antibacterial activity. This study was carried out following the method of Kirby-Bauer et al (1966). The pathogenic and resistant bacteria used for this study were: *Vibrio cholerae* Inaba, *Enterococcus faecalis* ATCC 29252, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Streptococcus mutans* ATCC 25175, multidrug resistant *Pseudomonas aeruginosa* (MDR-PA), and methicillin-resistant *Staphylococcus aureus* (MRSA). Every extract was dissolved in dimethyl sulfoxide (DMSO) as a test solution. Ten (10) µL (5% b/v) of this sample was dropped onto one piece of sterile disk paper (6 mm) placed on the surface of sterile agar media in a Petri disk. Paper disk containing chloramphenicol was a positive control. After incubation at 37°C for 24 hours, the inhibition diameter zone was measured.

The testing method for *M. tuberculosis* H37Rv bacteria was performed by agar diffusion method of Lowenstein Jensen (LJ) medium (Ministry of Health Indonesia 2014). The samples were prepared at concentration of 0.025%, 0.05% and 0.1% (b/v) in 5 mL of the medium, while LJ medium was prepared by dissolving 7.5 g of LJ medium in 120 mL distilled water containing 2.4 mL of glycerol and sterilized with autoclave at 121°C for 15 minutes. Duck eggs were washed then immersed in 70% ethanol for 15 min, broken, then blended in a sterile blender for 30 seconds to one minute. Aseptically mix the 1000 mL of egg suspension with the sterile LJ medium then cooled to 50-60°C, avoiding air bubbles. Microbe suspensions were made in distilled water and adjusted to a 0.5 McFarland's standard. The suspension was homogenized with vortex and allowed to settle down for 15 min. Next, each tube with 0.2-0.4 mL (2-4 drops) of the suspension was inoculated, then distributed over the surface and incubated at 37°C for 6-8 weeks. The culture observed within 5 to 7 days after inoculation and once a week thereafter for up to 8 weeks.

Isolation and characterization of secondary metabolites. The crude ethyl acetate (EtOAc) extract (38.58 g) of fungus IB141 was column chromatographed on silica gel using an increasing amount of MeOH in CH_2Cl_2 and fractions obtained were checked by thin layer chromatography (TLC), then the fractions having similar TLC pattern were combined to give four fractions (F1-F4). The second fraction (F2) was rechromatographed on silica gel eluted with CH_2Cl_2-MeOH (0.3:4.7) to give 7 subfractions (F21-F27). Subfractions F22 (27.7 mg), F23 (30.1 mg) and F26 (37.8 mg) were chromatographed on high performance liquid chromatography (HPLC) (C_{18}-reversed phase) eluted with MeOH-H_2O with 0.1% TFA at flow rate: 1 mL min^{-1} (from 10 to 100% MeOH, 30 min). HPLC analysis was performed with an UltiMate^{TM} 3000 UHPLC system and column C_8, 4.6 x 150 mm, 5 µm. The major peaks were compared to those of library hits of UV spectra data in Institute of Pharmaceutical Biology and Biotechnology, Heinrich Heine University Düsseldorf, Germany.

Results

Antibacterial activity. The antibacterial activity of ethyl acetate extracts of fungus IB141 toward all above pathogenic and resistant bacteria in agar medium showed significant activity against *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and methicillin-resistant *S. aureus* (MRSA) with the inhibition zone diameter more than 12 mm (Table 1).

Table 1

Zone of inhibition of the fungi-derived extracts against several pathogenic bacteria (mm)

Fungi extract	*Microorganism[a] (mm)*						
	VC	*EF*	*MDR-PA*	*SM*	*PA*	*MRSA*	*SA*
IB101	8.66	10.03	8.77	8.61	11.54	9.56	10.29
IB102	6.64	6.75	-	10.30	8.44	-	9.02
IB121	9.37	9.63	9.06	8.68	14.91	10.42	10.89
IB131	6.85	7.06	-	7.18	10.04	-	7.26
IB141	11.34	11.11	11.72	9.63	12.65	12.22	14.06
IB142	7.36	7.51	-	7.93	7.86	7.53	7.12
IB151	6.76	-	-	-	9.07	-	7.92
IB161	6.84	-	-	-	8.32	6.44	7.49

[a]VC = *Vibrio cholerae* Inaba, EF = *Enterococcus faecalis* ATCC 29252, MDR-PA = multi drug resistant *Pseudomonas aeruginosa,* PA = *Pseudomonas aeruginosa* ATCC 27853, SM = *Streptococcus mutans* ATCC 25175, MRSA = methicillin-resistant *S. aureus*, SA = *Staphylococcus aureus* ATCC 25923.

The concentration at 0.025, 0.05 and 0.1% (Table 2) show also strong inhibition (no growth) toward *M. tuberculosis* H37Rv in the LJ medium. Inhibitory activity for *M. tuberculosis* H37Rv was displayed by producing cream-colored buff, rough colonies after 2-3 weeks inoculation in LJ medium (Velayati & Farnia 2016). The growth of *M. tuberculosis* H37Rv colonies on extract of fungus IB141 (concentrations at 0.1%, 0.05% and 0.025%) was displayed in Figure 1.

Table 2

The antituberculosis activity against *Mycobacterium tuberculosis* H37Rv of fungi derived extracts from marine sponge *A. ingens*

Fungi extract	*Concentration*		
	0.1 %	*0.05 %*	*0.025 %*
IB101	Cont	3+	3+
IB102	2+	2+	3+
IB121	2+	2+	3+
IB131	2+	3+	3+
IB142	2+	3+	Cont
IB161	Cont	3+	3+
IB141	-	-	-
IB151	-	-	+1

- = no growth; 1+ = 20-100 colonies; 2+ = 100-200 colonies; 3+ = 200-500 colonies, Cont = contaminated.

A B C

Figure 1. Growth of *M. tuberculosis* H37Rv colonies on extract of fungus IB141 from marine sponge *A. ingens* at some concentrations: A = concentration of 0.1 %; B = concentration of 0.05%; C = concentration of 0.025%.

Isolation and characterization of secondary metabolites of fungus IB141. In this study, we tried to isolate the components of crude ethyl acetate extract by SiO_2 column chromatography and eluted by step gradient polarity elution using an increasing amount of MeOH in EtOAc and yielded four fractions. The second fraction (F2) was rechromatographed on silica gel eluted with CH_2Cl_2-MeOH (0.3:4.7) to give 7 subfractions and no pure compounds could be isolated. Further isolation attempts by using HPLC were not successful but the peak of the ultraviolet spectrum at each retention time compared to those of database available at library hits of UV spectra data at Institute of Pharmaceutical Biology and Biotechnology, HHU-Düsseldorf, Germany were identified as palitantin (1) (38.91 min, λ_{max} at 230.3 nm) for F22 fraction, citreodrimene A (2) (36.98 min, λ_{max} at 225.1 and 272.6 nm) for F23 fraction, septicine (3) (13.04 min, λ_{max} at 208.4 and 369 nm) and ochratoxin A (4) (28.11 min, λ_{max} at 214.6 and 332.7 nm) for F26 fraction (Figures 2 and 3).

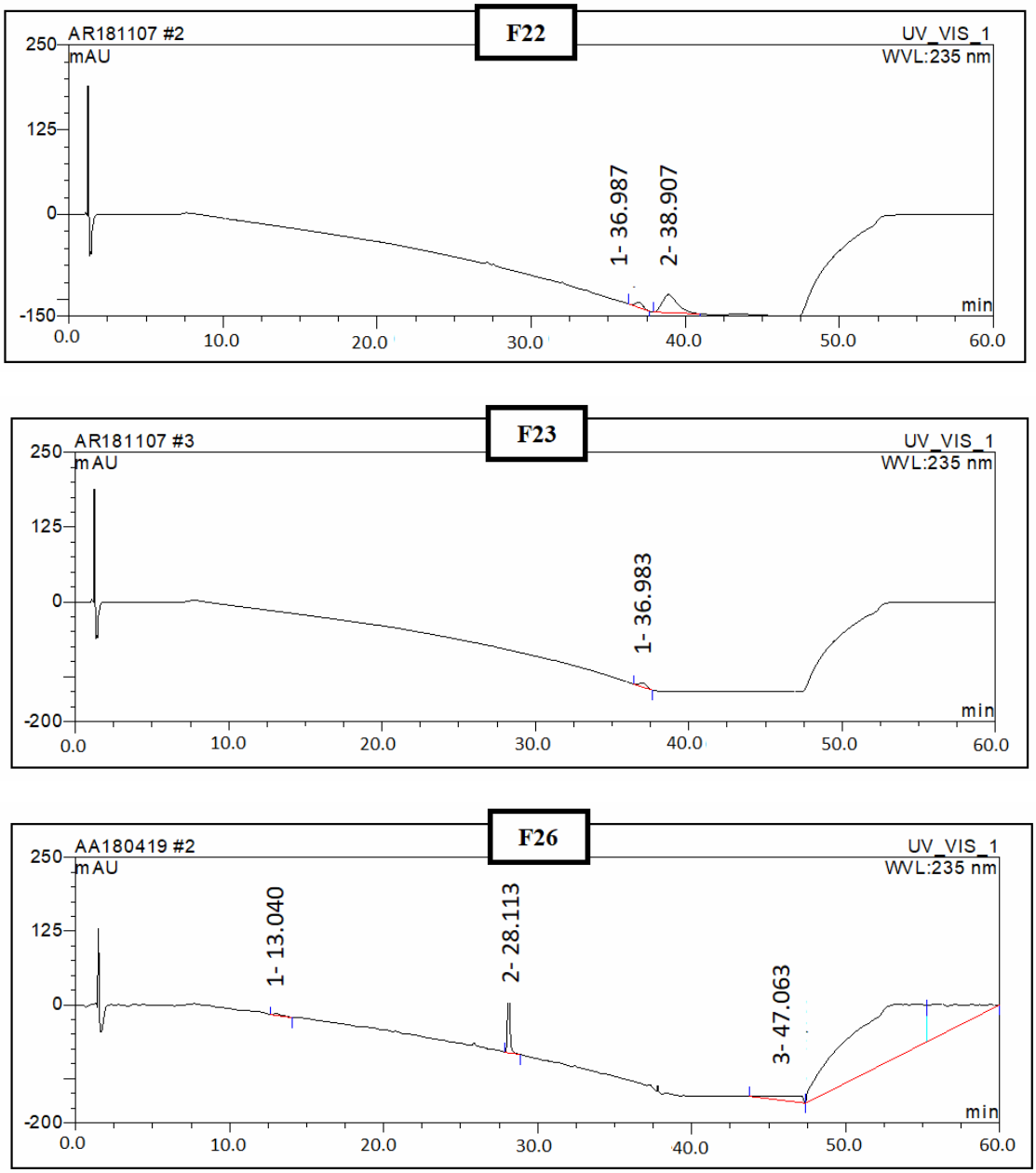

Figure 2. HPLC chromatogram of fractions F22, F23 and F26.

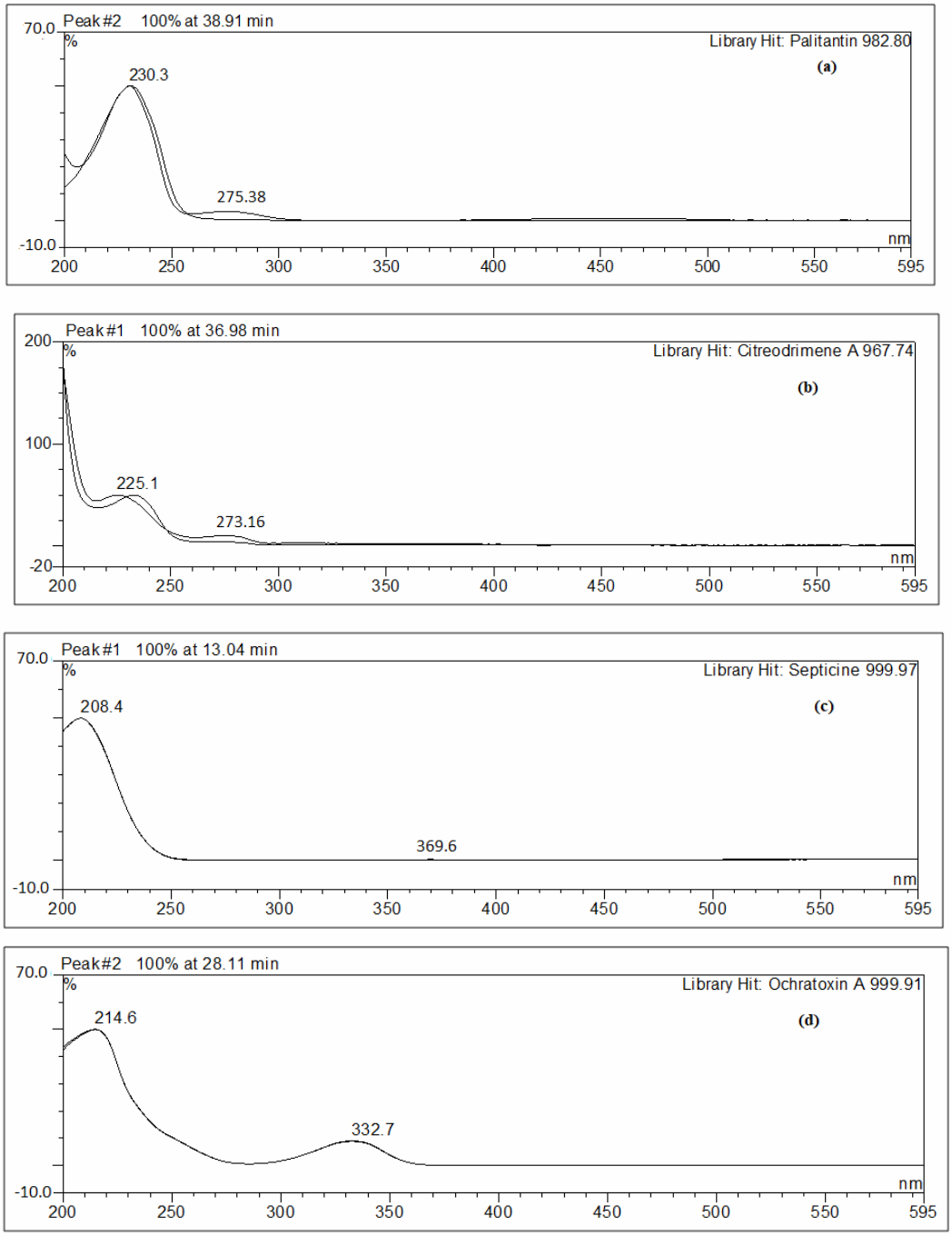

Figure 3. UV spectra of main peaks from fractions F22, F23 and F26: Library hit of UV spectra of the compound in retention time: (a) 38.91 min (palitantin), (b) 36.98 min (citreodrimene A), (c) 13.04 min (septicine), (d) 28.11 min (ochratoxin A).

Discussion. The marine sponge *A. ingens* was one of the endemic sponges found along Indonesian coastline. In our previous work, 8 symbiotic fungi were isolated from this sponge and the fungus IB141 was identified as *Aspergillus ochraceus* (Handayani & Aminah 2017; Aminah et al 2019). The best antibacterial activity of the fungus IB141 has been isolated by using HPLC. Palitantin (1), citreodrimene A (2), septicine (3) and ochratoxin A (4) were identified based on database available at library hits of UV spectra data at Institute of Pharmaceutical Biology and Biotechnology, HHU-Düsseldorf, Germany. (Figure 4). Wavelengths of these compounds have also been reported by previous studies (Van et al 1965; Rusman 2006; Uzma et al 2018; Ola et al 2018). These main peaks were responsible for the potential for antibacterial activity.

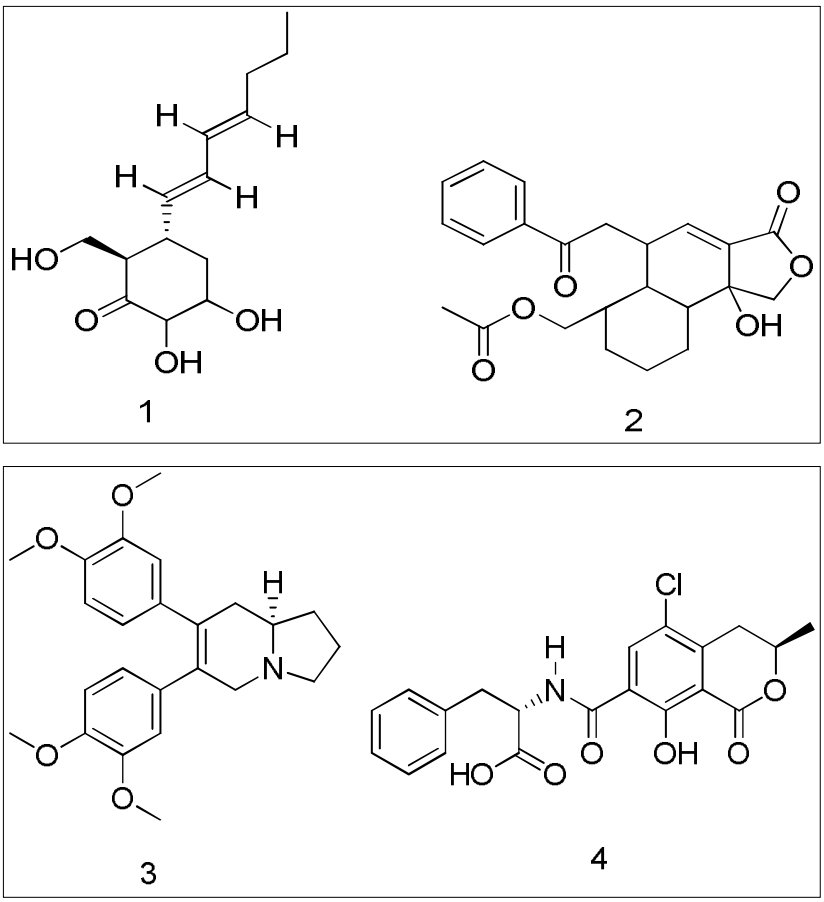

Figure 4. Chemical structures of palitantin (1), citreodrimene A (2), septicine (3) and ochratoxin A (4).

Information about antibacterial activity of the secondary metabolite of *A. ochraceus* from marine sponge *A. ingens* was limited. Several isolates of *A. ochraceus* from a variety of sources have been isolated and resulted in the isolation of toxic compound ochratoxin A (Hesseltine et al 1971; Moore et al 1974). Meanwhile, palintatin was found in a different genus, *Aspergillus fumigatiaffinis*, an endophytic fungus from the medicinal plant *Tribulus terrestris*. Palitantin was found to inhibit the growth of *E. faecalis* UW 2689 and *Streptococcus pneumoniae* with MIC value of 64 µg mL^{-1} (Ola et al 2018). Citreodrimene A from *Penicillium citreonigrum* showed a weak antimicrobial activity toward *B. subtilis* (Rusman 2016). Septicine from *Tylophora indica* displayed strong antibacterial activity against *B. subtilis*, *S. aureus*, *Micrococcus luteus* and *Pseudomonas aeruginosa* (Reddy et al 2009). Besides antibacterial activity, the above compounds had also antifungal, anti-inflammatory (Lee et al 2011), antiprotozoal (Fuska et al 1970), and cytotoxic activities against the L5178-Y (mouse T cell lymphoma), HeLa (human cervix carcinoma) cell lines (Rusman 2006).

In this study, *A. ochraceus* isolated from marine sponge *A. ingens* was able to produce the same secondary metabolites from different environments. Uniquely, the antibacterial activity of the extract from *A. ochraceus* in this study had the ability to inhibit *M. tuberculosis* H37Rv, which can appear as a productive source of new antituberculosis agents. Attempts to isolate the constituents of *A. ochraceus* in a pure form is in progress.

Conclusions. Fungus IB141 was isolated from marine sponge *Acanthostrongylophora ingens.* The fungus was identical to *Aspergillus ochraceus*. The ethyl acetate extract of IB141 was found to be most active against *Vibrio cholerae* Inaba, *Enterococcus faecalis* ATCC 29252, MDR-PA, *Pseudomonas aeruginosa*, MRSA, *Staphylococcus aureus* ATCC 25923 and *M. tuberculosis* H37Rv. Further study is recommended to isolate and identify

the active compounds that are responsible for the inhibition of the growth of some testing pathogenic bacteria.

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