

# *In vitro* Inhibitory Activity of Ethyl Acetate Extract of Symbiotic Bacteria Isolated from the Marine Sponge *Haliclona fascigera* against Multidrug Resistant Organism (MDRO)

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## ABSTRACT

Increased use of antimicrobial irrational human and veterinary medicine causes increasing emergence of antibiotic resistant organisms or multidrug resistant organisms (MDRO). MDRO are common bacteria that have resistance to multiple types of antibiotics. The objective of the study was to determine the presence of antibacterial activity in the 26 ethyl acetate extracts of symbiotic bacteria from the marine sponge *Haliclona fascigera* against MDRO. The MDRO employed were Vancomycin-resistant *Enterococcus* sp (VRE), extended-spectrum-beta ( $\beta$ )-lactamase gram-negative organisms (ESBL), Carbapenem resistant *Enterobacteriaceae* (CRE) and Multi resistant *Acinetobacter baumannii* (MRAB). The susceptibility of MDRO against the extracts was determined using the disk diffusion method. The zone of inhibition was measured and expressed in millimeters. The results showed that nine ethyl acetate extracts that considered active to VRE and ESBL. The strongest inhibition activity was found in extract of *Micrococcus* sp.1 (M2R2) with MIC values of 0.625% against VRE.

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## INTRODUCTION

Antimicrobial agents or antibiotics are used to prevent, control and reduce the occurrence of the infection. Antibiotics are semi-synthetic or synthetic substances produced by the natural metabolic processes of some microorganisms that can inhibit or destroy other microorganism (Basri & Nor, 2014). The increasing irrational use of antimicrobials in humans and veterinary medicine has consequently increased the emergence of antibiotic resistant organisms or multidrug resistant organisms (MDRO). People infected MDRO are more likely to have longer and more expensive hospital stays, and may be more likely to die as a result of the infection. When the drug of choice for treating their infection doesn't work, they require treatment with second- or third-choice drugs that may be less effective, more toxic, and

more expensive. This means that patients with an antimicrobial-resistant infection may suffer more and pay more for treatment. Today MDRO were considered to be one of the major health problem in the world (Baig, *et al.*, 2015). The United States Center for Disease Control and Prevention (CDC) and European Centre for Disease Prevention and Control (ECDC) came up with a consensual surveillance definition for MDRO, which states, "acquired non-susceptibility to at least one agent in three or more antimicrobial categories" (Magiorakos *et al.*, 2012). Recently, bacterial resistance to different antibiotics has raised dramatically leaving physician with few therapeutic options. Methicillin resistant *Staphylococcus aureus* (MRSA), extended-spectrum- $\beta$ -lactamases (ESBL) producing enterobacteria and vancomycin resistant enterococci (VRE) have become common hospital problems. Study of Mwanri and AlSaleh (2014) showed that from 758 organisms were isolated in Intensive Care Unit Of King Fahad Hofuf Hospital, Saudi Arabia, 90% of which were gram negative, and 10% were gram positive. While half of the patients had one organism, the other half had multiple organisms.

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The most frequent type of isolated organisms was *Acinetobacter* species (29.3%), *Pseudomonas aeruginosa* (21.6%), *Klebsiella pneumonia* (7%), ESBL producing *Klebsiella pneumonia* (6.7%), and *Methicillin Resistant Staphylococcal aureus* (MRSA) 5%.

Marine sponges have been the focus of many studies as they have proved to be a rich source of biologically active secondary metabolites (Edrada, *et al.*, 2000). These compounds may serve as a defense strategy to escape from predators, thus playing a crucial role in sponge survival in the marine ecosystem. Since the discovery of spongothymidine and spongouridine in the early 50's, a great number of biologically active compounds were isolated from marine sponges and their associated microorganisms (Graca, *et al.*, 2015). This continuous study is one of the efforts in exploring marine natural resources in the search for new antibiotic compounds at once an inventory of antibiotic activity derived from microbial symbioses with marine sponge derived from West Sumatra, Indonesia (Handayani, *et al.* 2015a; Handayani, *et al.* 2015b, Handayani, *et al.* 2016).

## MATERIAL AND METHODS

### Sponge material, isolation and cultivation of bacteria associated with marine sponge

Samples sponge that is used and the stages of the research method for isolation and cultivation of symbiotic bacteria have been carried out as it was written in previous studies (Handayani, *et al.* 2015).

### Antibiotic Susceptibility Test

The pathogenic bacteria Vancomycin resistant *Enterococci* spp (VRE), extended spectrum beta ( $\beta$ ) lactamase gram-negative organisms (ESBL), Carbapenem resistant enterobacteriaceae (CRE) and Multi resistant *Acinetobacter baumannii* (MRAB) were provided by the Laboratory of Microbiology, Public Hospital of M. Djamil Padang, West Sumatra, Indonesia. Antibiotic susceptibility of the MDRO was determined by disk diffusion technique using a commercially available disc (Oxoid<sup>®</sup>-CLSI, 2012). The antimicrobial sensitivity of the test strains to thirteen antibacterial drugs was done using the Kirby Bauer disk diffusion method (Bauer *et al.*, 1966). The antibiotic disks used were ceftazidime 30  $\mu$ g, cefotaxime 30  $\mu$ g, ceftriaxone 30  $\mu$ g, cefoperazone 30  $\mu$ g, ciprofloxacin 5  $\mu$ g, levofloxacin 5  $\mu$ g, ofloxacin 5  $\mu$ g, gentamycin 10  $\mu$ g, amikacin 30  $\mu$ g, piperacillin 100  $\mu$ g, ticarcillin 75  $\mu$ g, meropenem 10  $\mu$ g, and imipenem 10  $\mu$ g. The sterile nutrient agar poured into the plates. Further, 0.1 ml bacterial suspension was spread onto 20 ml of sterile agar plates by using a sterile *cotton bud*. The plates were allowed to dry before applying antibiotic disc. Then, some commercially available antibiotic discs were gently and firmly placed on the agar plates, which were then left at room temperature for 1 hour to allow diffusion of the antibiotics into the agar medium. The plates were then incubated at 37°C for 24 hours. If an antimicrobial activity was present on the plates, it was

indicated by an inhibition zone. The diameter of the inhibition zones was measured in millimeter at 24 hours using a scale. The zones of inhibition were measured and the organisms identified as sensitive or resistant based on standard criteria. ESBL production was detected by double disc synergy test. Synergy was determined between a disc of ceftazidime clavulanic acid and a disc of each third generation cephalosporin test antibiotic placed 15 mm apart on lawn culture of the isolate under test on Mueller Hinton agar plates. The test organism was considered to produce ESBL if the zone size around the antibiotic disc increased towards the ceftazidime clavulanic acid disc. The increase occurs as the clavulanic acid present in the ceftazidime clavulanic acid disc inactivates the ESBL produced by the test organism (Shaikh, *et al.*, 2015).

### Antibacterial Assay by Disc Diffusion Method

The disc diffusion assays were used with some modification to determine the antibacterial effect of ethyl acetate extracts of symbiotic bacteria against MDRO. Muller Hinton Agar (MHA) (Merck, Germany) was used to prepare the culture medium and autoclaved at 121°C for 15 min. Briefly, plates (14-cm diameter) were prepared with 14 ml MHA inoculated with 0.1 ml of bacterial suspension (0.1 ml of 0.5 McFarland Standard). The extracts were dissolved in dimethyl sulfoxide (DMSO, 15  $\mu$ L).

Sterile paper discs (6 mm in diameter) were impregnated with 0.01 ml 5% concentrations of extract placed onto MHA. The plates were incubated at 37°C overnight. Negative controls were prepared using the same solvent employed to dissolve the bacteria extract. Chloramphenicol 0.3%, Amikacin 30  $\mu$ g, Ciprofloxacin 5  $\mu$ g and Gentamycin 10  $\mu$ g antibiotic disc were tested in the same conditions as positive controls. Inhibition zones in mm (included disc paper diameter) around discs were measured. The antibacterial activity was expressed as the diameter of inhibition zones produced by the extract against test microorganisms. The experiment was repeated in duplicate and the mean of diameter of the inhibition zones was calculated.

### Determination of Minimum Inhibitory Concentration

The ethyl acetate extracts of symbiotic bacteria that showed greatest diameter of inhibition zone were screened to determine minimum inhibitory concentrations (MIC) by agar diffusion method. The concentration of extract were 5%, 2.5%, 1.25% and 0.625%. MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial strain.

### Identification of Isolates

Nine extracts of bacterial symbionts which showed high level of inhibition zone against MDRO were selected for further characterization and identification based on macroscopic and microscopic evaluation, gram staining, and biochemical assay to investigate the genus. All bacteriological isolates were examined and confirmed by biochemical tests according to *Cowan*

and Steel's Manual of Identification of Medical Bacterial Third Edition (1993).

## RESULTS AND DISCUSSION

MDRO used in this study has been conducted resistance test to some antibiotics. The test was important to prove that the bacteria used is MDRO. The test results of bacterial resistance of VRE, CRE, and MRAB showed sensitive only to the antibiotic fosfomycin and ciprofloxacin. ESBL- *Pseudomonas aeruginosa* showed resistant to all antibiotics.

The ethyl acetate extracts of bacterial symbionts were tested its bioactivity against microbial pathogens MDRO by agar diffusion method. Antibacterial activity test is done using a 5 % concentration of the extract in dimethylsulfoxide (DMSO). The antibacterial activity of the extracts was assessed from the results of measurements of clear area is formed. Clear area that formed was measured with calipers to the accuracy of 0.05 mm

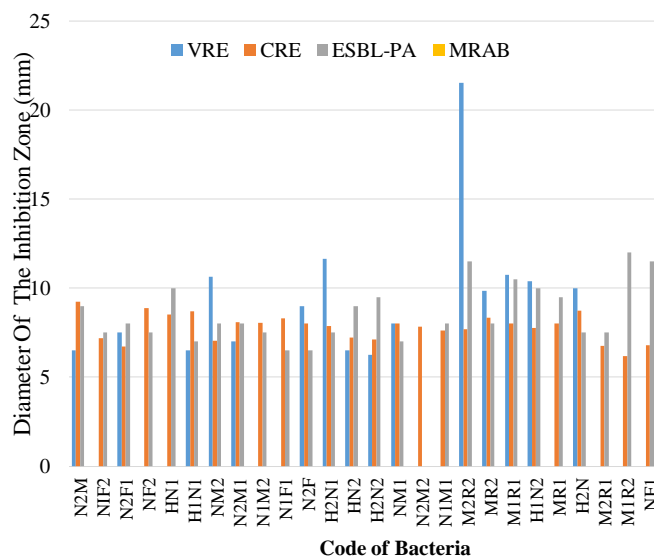
Based on the test results of the antimicrobial activity of the extract 26 bacterial isolates in Table 1, nine isolates showed growth inhibitory activity against MDRO. The extracts are M2R2, NF1, H2N1, H2N, M1R1, HN1, H1N2, NM2, and M1R2 with inhibition zones > 10 mm against VRE, CRE and ESBL. But none extracts can inhibit the growth of MRAB.

**Table 1:** Antibacterial activity of ethyl acetate extracts of symbiotic bacteria from marine sponge *H. fascigera* against MDRO.

No	Code of Bacteria	Diameter of the inhibition zone ± standard deviation			
		VRE	CRE	MRAB	ESBL
1	N2M	6.50±0.71	9.25±1.06	-	9.00±1.41
2	N1F2	-	7.18±1.17	-	7.50±0.71
3	N2F1	7.50±2.12	6.73±1.03	-	8.00±0.00
4	NF2	-	8.88±0.53	-	7.50±0.71
5	HN1	-	8.50±0.71	-	10.00±1.41
6	H1N1	6.50±0.71	8.68±0.46	-	7.00±0.00
7	NM2	10.63±0.88	7.05±0.64	-	8.00±0.00
8	N2M1	7.00±1.41	8.10±1.56	-	8.00±0.00
9	N1M2	-	8.05±0.07	-	7.50±0.71
10	N1F1	-	8.30±0.28	-	6.50±0.71
11	N2F	9.00±1.41	8.00±0.00	-	6.50±0.71
12	H2N1	11.65±0.92	7.88±0.18	-	7.50±0.71
13	HN2	6.50±0.71	7.23±0.11	-	9.00±0.00
14	H2N2	6.25±0.35	7.10±0.14	-	9.00±0.71
15	NM1	8.00±1.41	8.00±0.00	-	7.50±0.00
16	N2M2	-	7.83±0.95	-	-
17	N1M1	-	7.63±0.53	-	8.00±0.00
18	M2R2	21.50±0.71	7.68±0.95	-	11.50±0.71
19	MR2	9.83±0.60	8.33±0.11	-	8.00±1.41
20	M1R1	10.75±0.35	8.00±1.06	-	10.50±0.71
21	H1N2	10.38±0.53	7.75±0.35	-	10.00±1.41
22	MR1	-	8.00±1.41	-	9.50±2.12
23	H2N	10.00±1.41	8.73±1.80	-	7.50±0.71
24	M2R1	-	6.75±1.06	-	7.50±0.71
25	M1R2	-	6.18±0.25	-	12.00±0.00
26	NF1	-	6.80±0.28	-	11.50±0.71

Arora & Bhardwaj (1997) has stated that the antibacterial activity can be classified based on the level of sensitivity according to the size of diameter of inhibitory zone without reducing the diameter of the paper disk. High sensitivity is obtained if the diameter of inhibition zone reached > 12 mm.

Intermediate sensitivity category declared if the diameter of the zone is approximately 9-12 mm. Category low sensitivity when the diameter of the zone is 7-9 mm and resistant if ≤ 6 mm. The greatest activity of extract was found on bacteria code of M<sub>2</sub>R<sub>2</sub> against VRE that classified a high sensitivity level with the diameter inhibition zone of 21.50±0.71 mm (Fig. 1).



**Fig. 1:** Antibacterial activity of ethyl acetate extract of symbiotic bacteria against MDRO.

The Extract with the highest antibacterial activity is then determined the Minimum Inhibitory Concentration (MIC) by the method of diffusion agar. MIC determination is done by using multiple concentrations of 5%; 2.5%; 1.25%; and 0.625%. The test results showed inhibition zones of 18.5 ± 0.35 mm; 10.1 ± 1.07 mm; 8.25 ± 0.18 mm; and 6.125 ± 0.09 mm, respectively (Table 2).

**Table 2:** Antibacterial activity of ethyl acetate extract of symbiotic bacteria M<sub>2</sub>R<sub>2</sub> againsts Vancomycin Resistant *Enterococcus faecalis* (VRE) in different concentration.

Concentration	Diameter of Inhibition Zone (mm)		Mean of Inhibition Zone ± Standard Deviation
	Paper Disk 1	Paper Disk 2	
5.00%	18	19	18.5±0.35
2.50%	10	10.2	10.1±1.07
1.25%	8	8.5	8.25±0.18
0.63%	6	6.25	6.125±0.09

It showed that the lowest concentration of bacterial extract M<sub>2</sub>R<sub>2</sub> that still give the antibacterial activity was 0.625 % (Fig. 2). Nine isolates bacterial symbionts that have antibacterial activity against MDRO were identified by examination of morphological characters macroscopically and microscopically, gram staining and biochemical tests to determine the bacterial genus. Identification of the bacteria refers to a book Cowan and Steel Manual of Medical bacterial identification Third Edition (1993). Biochemical test was done to determine the type or genus of bacteria isolates associated with sponge of *H. fascigera*.

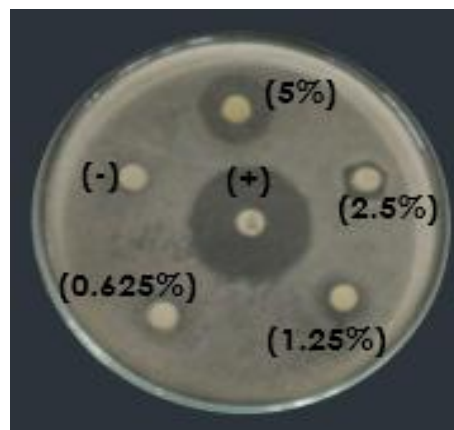


Fig. 2: Antibacterial activity of ethyl acetate extract of symbiotic bacteria MR<sub>2</sub> against Vancomycin Resistant *Enterococcus faecalis* (VRE).

Table 3: Morphological and biochemical characterization for the identification of the selected marine bacterial strains.

Code of Bacteria	Nutrient Agar	Color of colony	Gram	TSIA	Gas	H <sub>2</sub> S	Catalase	Oxidase	Motility	Indol	Urea	Citrate	Lactose	Glucose	Sucrose	Mannitol	Methyl Red (MR)	Voges Proskauer (VP)	OF	Identity	
M <sub>2</sub> R <sub>2</sub>	+	YellowCoccus	+	Y/y	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Micrococcus</i> sp.1
NF <sub>1</sub>	+	YellowCoccus	+	Y/y	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Micrococcus</i> sp.2
H <sub>2</sub> N <sub>1</sub>	+	YellowBasil	+	Y/y	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	<i>Bacillus</i> sp.1
H <sub>2</sub> N	+	YellowBasil	+	Y/y	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	<i>Bacillus</i> sp.2
M <sub>1</sub> R <sub>1</sub>	+	YellowBasil	+	Y/y	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	<i>Bacillus</i> sp.3
HN <sub>1</sub>	+	YellowBasil	+	Y/y	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	-	<i>Bacillus</i> sp.4
H <sub>1</sub> N <sub>2</sub>	+	YellowLong Basil	+	Y/y	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Corynebacterium</i> sp.1
NM <sub>2</sub>	+	YellowLong Basil	+	Y/y	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	<i>Corynebacterium</i> sp.2
M <sub>1</sub> R <sub>2</sub>	+	WhiteCoccus	+	Y/y	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	-	<i>Staphylococcus aureus</i>

+ : positive ; - : negative ; Y/y : The medium is yellow and the color of medium does not change after inoculated of bacteria and 24 hours incubation at 37 °C.

These tests are used because there are some bacteria that have special properties when reacted with certain biochemical test that can be the specificity or specific genus. Some biochemical tests were conducted as TSIA (Triple Sugar Iron Agar), gas, H<sub>2</sub>S, catalase, oxidase, motility, IMViC (Indole, Methyl Red-Voges Proskauer/MR-VP, Citrate), urea, lactose, glucose, sucrose, mannitol, nitrate and gelatin.

The results show that the identification of the four genera classified as *Bacillus* sp. hereinafter coded as *Bacillus* sp 1, *Bacillus* sp 2, *Bacillus* sp 3 and *Bacillus* sp 4, two bacteria as *Corynebacterium* sp 1 and *Corynebacterium* sp 2. Other two bacteria classified as *Micrococcus* sp.1 and *Micrococcus* sp 2 and one other bacteria as *Staphylococcus aureus* (Table 3). It has been reported that microorganisms play a role in the biological life of the sponge as they are associated with a lot of sponge in the intracellular and extracellular or both. Around 40% of sponge biomass is thought to contain bacterial community. Some studies have shown that the symbiont has a role in production of bioactive compounds that function in ecological adaptation sponge (Sfanos *et al.*, 2005; Proksch, *et al.*, 2003). Based on the research result, it

has been proven that the marine sponge *H. fascigera* containing a large amount diverse of bacteria and fungi. The results of antibacterial bioactivity screening of extracts of microorganisms have proved that they are capable of producing secondary metabolites that are needed to overcome the problem of the treatment of a resistant bacterial infection as MDRO.

## CONCLUSIONS

Infectious disease caused by MDRO was a growing problem in healthcare. Such infections are lead to patients should be treated longer thus resulting increase of medical expenses in the hospital. Therefore, effort is needed in the discovery of new antibiotics to overcome the MDRO.

From the results of this research, 26 symbiotic bacteria from marine sponge *H. fascigera* were isolated. 9 isolates of which can inhibit the growth of VRE, CRE and ESBL. It could also be concluded from this study that the symbiotic bacteria can be used as an important alternative to produce new antibiotics needed in overcome the infection caused by MDRO.

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